

METABOLISM AND TOXICITY OF BENZENE

By

Anders Tunek

Lund 1981

From the Institute of Environmental
Health, University of Lund, Sweden

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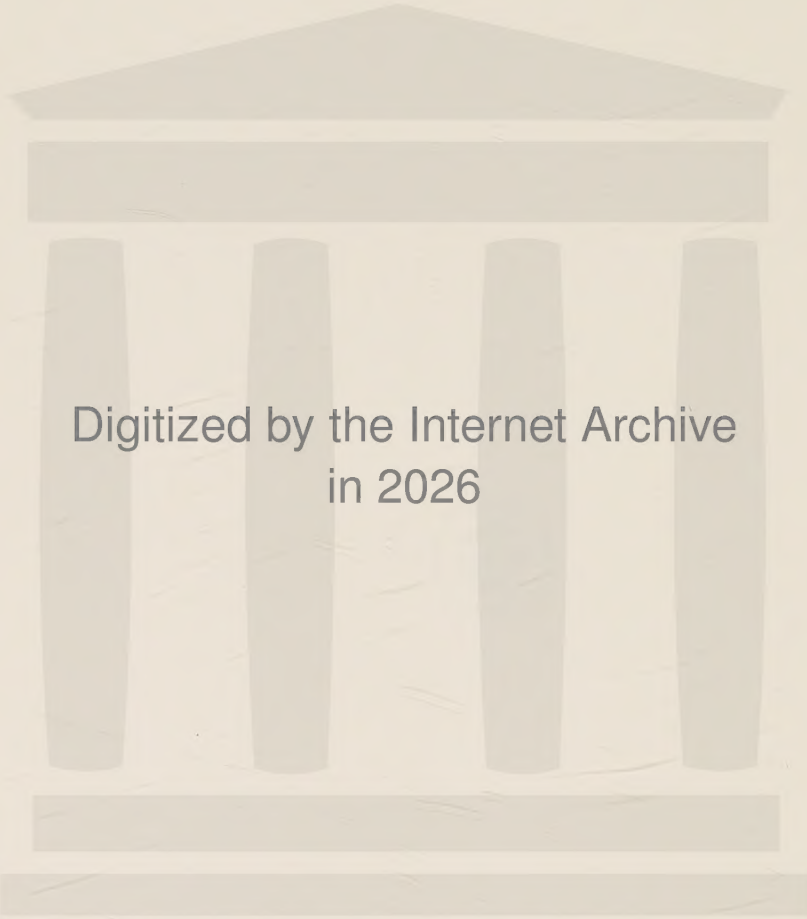
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PREFACE

I wish to express my sincere gratitude to all involved in the study:

Professor Maths Berlin, head of the Institute of Environmental Health, University of Lund, for placing facilities at my disposal and for many discussions on environmental and occupational health problems.

Professor Franz Oesch, head of the Section of Biochemical Pharmacology, University of Mainz, Germany, for giving me the opportunity to work at his institute for a year and a half. Professor Oesch's supervision, his enthusiastic attitude to science and his constant encouragement have been invaluable for me. Several members of his research group are also acknowledged for many discussions on scientific matters, and for making the time in Mainz a most enjoyable experience for me and my family.

Docent Bengt Jergil, Department of Biochemistry 1, University of Lund, for teaching, fruitful collaboration and many discussions through the years, and for most valuable help in making this thesis readable.

Cecilia Schelin, Department of Biochemistry 1, University of Lund, for fruitful and interesting collaboration during the last couple of years.

Dr. Tor Olofsson, Research Department 2, University Hospital, Lund, for excellent teaching on bone marrow biology and for very valuable collaboration.

Dr. Benkt Högstedt, Institute of Environmental Health, University of Lund, for collaboration and for making me aware of the potential of the micronucleus test.

Inger Möller for the excellent typing of this thesis and several other manuscripts. In addition, throughout my work I have taken advantage of her expert knowledge of the English language.

Finally, the rest of the staff at the Institute of Environmental Health, University of Lund, for the pleasant time we have spent together.

The investigations presented in this thesis were financially supported by the Medical Faculty of the University of Lund, the Swedish Cancer Society, the Swedish Medical Research Council, Deutscher Akademischer Austauschdienst, Stiftung Volkswagenwerk and the Swedish Natural Science Research Council.

This thesis is based on the following papers which will be referred to by their Roman numerals.

I Tunek, A. and Oesch, F.

Unique behaviour of benzene monooxygenase: Activation by detergent and different properties of benzene- and phenobarbital-induced monooxygenase activities.

Biochem. Pharmacol., 28:3425-3429, 1979.

II Tunek, A., Platt, K.L., Bentley, P. and Oesch, F.
Microsomal metabolism of benzene to species irreversibly binding to microsomal protein and effects of modifications of this metabolism.

Mol. Pharmacol., 14:920-929, 1978.

III Tunek, A., Platt, K.L., Przybylski, M. and Oesch, F.

Multistep Metabolic Activation of Benzene.

Effect of superoxide dismutase on covalent binding to microsomal macromolecules, and identification of glutathione conjugates using high pressure liquid chromatography and field desorption mass spectrometry.

Chem.-Biol. Interact., in press.

IV Tunek, A., Schelin, C. and Jergil, B.

Microsomal target proteins of metabolically activated aromatic hydrocarbons.

Chem.-Biol. Interact., 27:133-144, 1979.

V Tunek, A., Olofsson, T. and Berlin, M.

Toxic effects of benzene and benzene metabolites on granulopoietic stem cells and bone marrow cellularity in mice.

Submitted for publication to Toxicol. Appl. Pharmacol.

VI Tunek, A., Högstedt, B. and Olofsson, T.

Toxic effects of benzene and hydroquinone on bone marrow in mice.

Manuscript.

BENZENE METABOLISM AND TOXICITY: A REVIEW

Introduction

The hazards associated with exposure to benzene have been recognized by industrial toxicologists since before the turn of the century. Already in 1897 Santesson described chronic toxicity of benzene, and ascribed four deaths to benzene exposure (1). During the second decade of this century, Selling performed his classical experimental studies on the mechanism of benzene toxicity in bone marrow (2, 3). In 1922 Hamilton published an article entitled "The growing menace of benzene (benzol) poisoning in American industry" (4). In the next couple of years, steps were taken to reduce exposure to benzene, and in 1928 Hamilton could publish a second paper, "The lessening menace of benzol poisoning in American industry" (5). This title now seems overoptimistic, since the immense industrial development that followed dramatically increased the utilization of benzene. Even today, particularly in less wealthy countries, there is, unfortunately, clinical material in which to study benzene toxicity.

Sources, usage and properties of benzene

Benzene is a clear, colourless liquid with a rather pleasant odour and a boiling point of 80°C. The main hygienic problem with benzene is that it will easily evaporate and contaminate the air wherever handled, unless protective actions are undertaken.

The special properties of benzene as a solvent and

as a starting point for organic synthesis, together with its ability to replace lead as an anti-knocking agent in motor fuels, have made benzene a ubiquitous component of our chemical age (6). Benzene is used chiefly as an intermediate in producing other chemicals such as phenol, cyclohexane, styrene, detergents and pesticides, in the rubber and plastics industry and in chemical laboratories. Furthermore, most European motor fuels contain 1-16% benzene with an average of about 5%, while the average is 0.8% for US fuels (7, 8, 9).

During the last decades there has been a sharp increase in the production of benzene. In 1940, 0.46 million tons were produced in the United States, and in 1974 the production had risen to 4.6 million tons. Before 1940, benzene was produced commercially from coal. Today, however, benzene is mainly obtained from the petroleum industry, where it is produced from paraffinic hydrocarbons. Toluene and xylene are major impurities in commercial benzene, while phenol, thiophene, carbon disulfide, acetyl nitrile and pyridine may be minor contaminants (10).

Levels of exposure to benzene

Knowledge of the severe toxic effects of benzene has increased dramatically during the last couple of decades. As a result, actions to limit exposure to benzene have been undertaken in most industrial plants where this chemical is used. In some cases benzene has been replaced by other solvents as e.g. in the printing industry where today toluene usually is used instead of benzene. Despite the increased awareness of the toxic effects, benzene is still widely used; thus, the National Institute for Occupational Safety and Health (NIOSH) estimated that in 1974, 2 million workers in USA were potentially exposed to benzene (11). The threshold limit value for occupational exposure in Sweden is presently 5 ppm TWA (time weighted average), NIOSH has recommended 1 ppm in USA and the threshold

limit value in the Soviet Union is 1.6 ppm.

To determine exposure levels one needs a reproducible and sensitive analytical method. In many reports, old as well as relatively recent ones, exposure levels either have not been measured at all, or have been measured with unreliable methods. It seems clear, however, that in the early days of industrial development exposure levels well in excess of 1,000 ppm were common. The exposure levels of today are much lower, at least in developed countries. Table 1 shows benzene levels at some Swedish places of work where there was a potential risk for benzene exposure. These measurements were undertaken during the years 1975-1979. In countries with lower hygienic standards, exposure levels can still be high. Several reports from recent years describe severe benzene-induced toxicity, in some cases even leading to death (13, 14). In one of these studies, with four deaths due to leukemia, the exposure level was measured to be 210 ppm (15).

As stated above, benzene is a constituent of most motor fuels. This means that benzene is not only a problem of industrial plants, but also a general environmental pollutant. Some leakage must be expected when filling gasoline into cars and, furthermore, the combustion in the motors is incomplete (6). Air samples from a street with heavy traffic contained 0.007 ppm benzene, while exhaled air from individuals not occupationally exposed to benzene showed benzene levels of 0.001-0.002 ppm (16). In addition, cigarette smoke from one cigarette contains 64-107 μ g benzene, and exhaled air from smokers not occupationally exposed to benzene was found to contain up to 0.006 ppm benzene (17).

It is unknown to what extent, if at all, the low levels of benzene encountered in the urban environment have adverse effects on humans. As will be discussed in more detail below, however, it is a matter of concern that occupational exposure levels lower than 10 ppm appear to induce chromosome breaks and gaps in workers (17, 18, 19).

Table 1* Exposure to benzene at places of work in Sweden measured by personal sampling.

	Sampling time (min)	Exposure (ppm, average, range)
Road tanker drivers	45	0.4 (4.13 - 0.01)
loading	45	4.0 (45.5 - 0.03)
unloading	45	0.33 (2.9 - 0.01)
driving	45	0.026 (0.11 - <0.005)
Petrol station attendants	45	0.084 (0.31 - 0.02)
Seamen on coastal tankers	45	6.56 (22.8 - 0.33)
Coke workers	9-25	2.72 (12.0 - 0.16)
Automobile servicemen	10	0.052 (0.083 - 0.008)
Lumberjacks	8-10	0.29 (0.92 - 0.02)
Chemical laboratory	10	0.44 (2.0 - 0.04)
Manual cleaning of petrol tanks	4-8	195 - 18.7**
Petrol pump servicemen	6-10	0.41 (0.64 - 0.23)

* Based on references 9 and 12

** Stationary measurements 1 m from tank opening during manual unloading gave 527 ppm

Uptake of benzene by the organism

Benzene is rapidly absorbed through the alveolar walls when inhaled. This is illustrated by an experiment in which human volunteers were exposed to benzene (20). After 10 min. of exposure the concentration of benzene in the exhaled alveolar air was 25% of the exposure level and after 20 min. a concentration of about 40% was reached. This level then rose with about 3% per h. Several other studies have shown the overall retention of inhaled benzene in man to be around 50% (21, 22, 23).

There are certainly other routes of benzene uptake than via inhalation but these are of less practical importance. For instance, in rats benzene is rapidly absorbed from the gut (24). In some exceptional cases absorption through the skin might be important, despite the fact that benzene penetrates human skin less readily than e.g. toluene or ethyl benzene (25, 26, 27).

Distribution of benzene and its metabolites in the organism

In humans 25-50% of the benzene absorbed via inhalation will be eliminated unchanged by exhalation (23, 28, 29). The rest of the benzene will be distributed among the organs in a way that is determined by blood flow and fat content. The distribution coefficients between tissue and blood have been calculated to be 1.5 for organs rich in blood vessels (heart, brain etc.), 1.1 for skeletal muscles, 58.5 for adipose tissues (fat, yellow bone marrow) and 16.2 for red bone marrow. Theoretical half-lives for benzene are, in the same order, 1.7 min., 16.3 min., 32.4 h. and 2.55 h. (30, 31). Tissues with a high fat content often have a low blood flow. Hence a long time of exposure may be required before the benzene content in these tissues comes to equilibrium with the exposure level. Human volunteers exposed to a benzene atmosphere 8 h./day

for 5 days had a benzene concentration in exhaled breath that was twice as high 16 h. after the fifth day of exposure as 16 h. after only one day of exposure (32). This indicates that benzene can accumulate in depots if exposure occurs regularly.

When mice were injected subcutaneously with a single dose of benzene, the distribution of benzene was as follows: fat >> red bone marrow >> blood, liver > spleen. Quite interestingly, after the same injection of benzene, the metabolites of benzene were distributed differently: red bone marrow >> liver > blood > fat > spleen (33). This might be of toxicological importance since bone marrow is the main target organ for benzene toxicity. It should be noted, however, that whole-body autoradiography studies in mice did not indicate any accumulation of benzene metabolites in bone marrow (34).

The disposition of benzene in several organs and the concentrations of its metabolites in blood and bone marrow were investigated by Rickert et al. (35) in rats inhaling 500 ppm benzene. The steady state concentrations of benzene in blood, bone marrow and fat were 11.5, 37.0 and 164 $\mu\text{g/g}$, respectively. These steady state levels were achieved within 6 h. of exposure. Phenol was the main metabolite of benzene present in the bone marrow at early times. At later times, however, free catechol and, particularly, free hydroquinone reached much higher and relatively persistent concentrations than free phenol in this tissue.

Covalent binding of benzene metabolites to DNA has been observed in livers of rats exposed to a benzene atmosphere (36). Furthermore, binding to macromolecules in several organs (liver, brain, kidney, spleen, bone marrow and fat) was observed following subcutaneous injections of benzene in mice (37). Surprisingly, the covalent binding in these experiments was more than 10 times higher in the liver than in the bone marrow. Recently, Irons et al. (38) demonstrated covalent binding of benzene metabolites to macromolecules in per-

fused rat bone marrow after injection of benzene into the perfused organ. This indicates that the bone marrow itself can metabolize benzene to reactive metabolites.

Elimination of benzene and its metabolites

As mentioned above, 25-50% of the benzene retained by man when exposed via inhalation is eliminated unchanged via exhalation. In one experiment where human volunteers were exposed to 100 ppm for 5 h., 70% of the retained benzene was eliminated in the urine as phenol, hydroquinone or catechol or their conjugates. Only 0.2-0.4% of the benzene was excreted unchanged via the kidneys (23). In the rat 1% of a benzene dose was excreted into the bile (39).

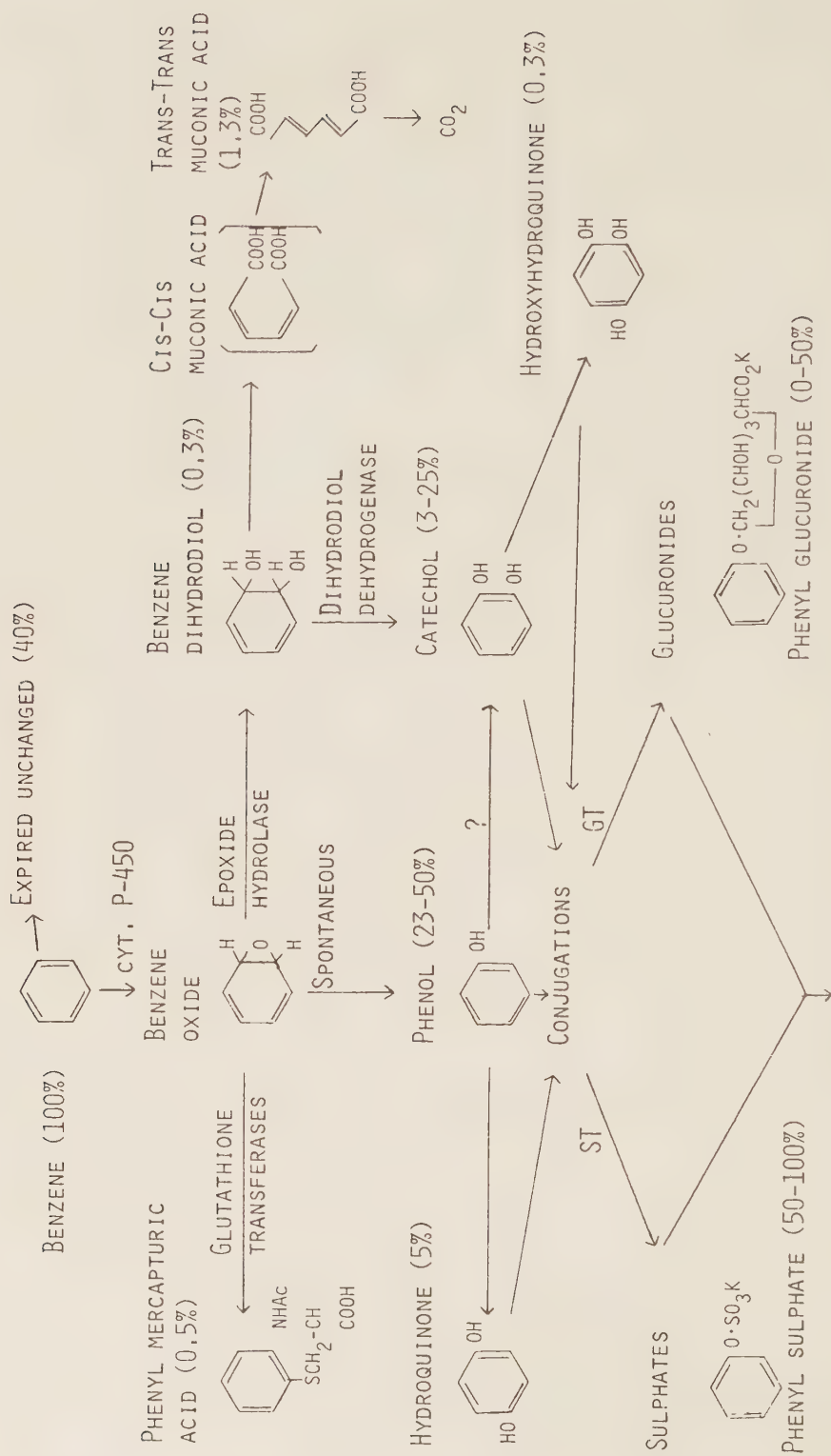
The elimination of benzene via exhalation in humans occurs in several phases. A number of rapid phases have an average apparent half-life of about 2.6 h., and some slower elimination phases give an apparent half-life of approximately 24 h. (32). In rats, the exhalation of benzene after inhalation exposure was biphasic. The first phase had a half-life of about 0.7 h., and the second one of about 13.1 h. (35). The different phases in the elimination process probably reflect the fact that benzene accumulates in different depots, which release benzene into the blood at different rates.

Biotransformation of benzene

A scheme summarizing the most important biotransformation pathways for benzene in mammals is shown in Fig. 1 (p. 8).

a. In vivo

The metabolism of benzene is a typical example of the two-phase detoxification mechanism: first the benzene molecule is hydroxylated once, twice or even three times and then the hydroxylated metabolite is conjugated with sulphate or glucuronic acid to form water-soluble compounds which are excreted into the urine.



ELIMINATED IN URINE

ST= SULPHATE

TRANSFERASE

GT= GLUCURONOSYL

TRANSFERASE

As will be seen, however, a few side tracks exist in the biotransformation scheme. The main metabolite of benzene is always phenol, which is excreted in the urine largely as a conjugate, but often also to some extent in the free form. The conversion of benzene to phenol in animals was first observed as early as 1867 (41).

The first major series of studies on the metabolic fate of benzene in animals was initiated in 1949 by Porteous and Williams (42, 43). They administered benzene orally to rabbits. Using chemical methods they could demonstrate that the principal metabolites in urine were ethereal sulphate and glucuronide conjugates of phenol, hydroquinone, catechol and hydroxyhydroquinone together with traces of trans-trans-muconic acid. Virtually all the phenolic metabolites were excreted as conjugates. The time-courses for the urinary elimination of the different metabolites were different. Thus, 95% of the phenol was excreted during the first two days. In comparison, only 60% of the catechol and hydroquinone was eliminated during the same period of time. The hydroxyhydroquinone elimination did not reach its maximum until the third day. It was therefore suggested that the dihydroxyphenols were subsequent hydroxylation products of phenol, and that the trihydroxyphenol was an even later oxidation product. As described below, the view today is that catechol is formed via benzene dihydrodiol rather than from phenol, and that trihydroxyphenol is formed from catechol rather than from hydroquinone.

More quantitative metabolic studies could be performed when radioactively labelled benzene became

Fig. 1. Biotransformation pathways for benzene in mammals. Modified after Rusch et al. (40).

available. When ^{14}C -benzene was administered orally to rabbits, 84-89% of the radioactivity was recovered in expired breath, urine, feces and body tissues. 43% of the radioactivity was recovered as unchanged benzene and 1.5% as $^{14}\text{C-CO}_2$ in the expired breath. Notably, the elimination of $^{14}\text{C-CO}_2$ started as late as 12-18 h. after benzene administration and continued for several days. The urine contained 34.5% of the radioactivity of which phenol accounted for 23.5%, catechol for 2.2%, hydroquinone for 4.8%, hydroxyhydroquinone for 0.3%, trans-trans-muconic acid for 1.3%, and L-phenylmercapturic acid for 0.5%. No free phenols were found in the urine. The feces and body tissues contained 5-10% of the dose (44, 45).

Parke and Williams (45) administered ^{14}C -phenol orally to rabbits in order to examine the mechanism of formation of the trans-trans-muconic acid. This acid was not formed from phenol and, therefore, benzene dihydrodiol was postulated to be an intermediate in benzene biotransformation. The results of this study further show that the ratio hydroquinone:catechol was 10:1 after administration of phenol and 2:1 after administration of benzene. Several subsequent studies have confirmed that catechol, if at all formed from phenol, is a minor hydroxylation product, while hydroquinone is the principal further hydroxylated metabolite of phenol (46, 47). Instead, catechol is probably mainly formed from benzene dihydrodiol through the action of dihydrodiol dehydrogenase, an enzyme present in the cytosolic fraction (48, 49). Benzene dihydrodiol was later reported to be present as its glucuronide in the urine of rabbits which had been given benzene intraperitoneally (50). The metabolism in vivo of hydroquinone and catechol in rabbits was examined by Garton and Williams (51, 52). They were not able to find any evidence for the addition of a third hydroxyl group to hydroquinone, while hydroxyhydroquinone could be demonstrated as a metabolite of catechol.

Snyder (53) investigated the metabolism of sub-

cutaneously injected benzene in mice. He administered doses ranging between 440-8,800 mg/kg and collected urinary metabolites over a period of 24 h. It was found that a mouse weighing 25 g could metabolize at the most 1 mmole benzene per day. The major metabolite was phenol, but traces of catechol were also identified. Of the phenolic metabolites, 50-65% were glucuronides, 26-38% sulphates and around 5% was free phenol.

The metabolism of benzene in humans seems to be essentially the same as in animals, although, for obvious reasons, it has not been studied in such detail. Teisinger et al. (23) exposed human volunteers to a benzene atmosphere of 100 ppm for 5 h. and found that 46% of the inhaled benzene was retained. Of the retained benzene 60% was excreted in the urine as phenol, 6% as catechol, and 2% as hydroquinone.

Generally, it seems that there are no striking differences between different species in the oxidative metabolism of benzene. The conjugation reactions, however, show more variation. Oehme (54) demonstrated that ^{14}C -phenol was conjugated almost exclusively with sulphate in the cat, while glucuronides dominated strongly in the pig. These results were confirmed by Capel et al. (46), who also showed that 17 other species, including man, formed both types of conjugates. Generally, sulphate conjugates dominate at low hydrocarbon doses, while glucuronides become more important at higher doses (7). This might be due to either limitations in the sulphate pool or to saturation of the sulphate transferases. Fasted rats had a much higher percentage glucuronides than fed animals (55), indicating that the amount of sulphate was the limiting factor in this case.

As already described, covalent binding of benzene metabolites to macromolecules has been demonstrated in vivo. Binding occurred to liver DNA in rats exposed to benzene via inhalation (36), and to macromolecules in several organs (liver, brain, kidney, spleen, bone marrow and fat) in mice exposed to benzene via subcutaneous injections (37).

b. In_vitro

The first attempts to study the metabolism of benzene in subcellular fractions were performed using relatively insensitive and unspecific colorimetric methods. Sakamoto et al. (56) studied benzene metabolism in rabbit liver homogenates. They were able to show that phenol was the principal metabolite, and that pyridine nucleotides were required for metabolism. They also used differential centrifugation to localize the active enzyme systems, but did not reach high enough centrifugal forces to isolate microsomes. Their conclusion was that the enzyme forming phenol from benzene was located in the 19,000 x g supernatant. Using similar colorimetric methods, Posner et al. (57) demonstrated that the conversion of benzene to phenol took place in microsomes isolated from rabbit or dog liver.

The introduction of radiolabelled benzene facilitated more detailed studies of benzene metabolism in vitro. Snyder et al. (58) used subcellular fractions of liver from rabbit, rat and mouse. Free phenol as well as sulphate and glucuronide conjugates were obtained in liver homogenates or 9,000 x g supernatants, whereas only free phenol could be demonstrated as a metabolite when isolated microsomes were used. Microsomes from mouse liver were considerably more active than those from rabbit or rat liver; the specific activities for mouse, rabbit and rat were 31, 1.8, and 3.5 nmol phenol formed/mg microsomal protein/h., respectively. Harper et al. (59) investigated the benzene hydroxylases in liver microsomes from hamster, rat and rabbit. They found V_{max} values of 9.29, 1.35, and 3.86 nmol phenol formed/mg protein/min. for the three species, respectively. In comparison, lung microsomes had V_{max} values of 3.16, 1.13, and 10.4, respectively.

Several pieces of evidence indicate that benzene is hydroxylated by the cytochrome P-450-dependent mixed function oxidase system. This enzyme system is present in the endoplasmic reticulum of most mammalian cell

types investigated (60, 61), and it has been extensively studied and characterized during the last two decades. This system has a remarkable lack of substrate specificity, probably largely due to the existence of several forms, constitutive and inducible, of cytochrome P-450 (62, 63). The experimental findings supporting the concept that benzene is hydroxylated by the cytochrome P-450 are e.g. that benzene forms a type 1 spectral change when added to microsomes, that O_2 and NADPH are required for the formation of phenol from benzene and that CO blocks this formation (64). Furthermore, cytochrome P-450 inducers will also enhance benzene hydroxylation and most cytochrome P-450 inhibitors will inhibit this reaction, as will be discussed in more detail in the next chapter.

Jerina et al. (65) obtained evidence suggesting that benzene oxide is an intermediate in the hydroxylation of benzene by showing that the microsomal metabolism of benzene oxide yielded the same major metabolites as did the in vivo metabolism of benzene. Metabolically formed benzene oxide could, however, not be isolated. Oesch et al. (66) showed benzene oxide to be a substrate for rat liver epoxide hydratase. The K_m of the reaction (32.5 mM) was unusually high. This might be due to an equilibrium reaction between arene oxide and oxepin. The equilibrium constant for this reaction is not known and, therefore, the effective concentration of the arene oxide in the incubation mixture might have been much lower. The rate of benzene dihydrodiol formation was in the same range as that of most other epoxide hydratase-mediated reactions, namely 24.5 nmol/mg protein/min.

Very little information is available on the metabolism of benzene in other organs than liver, although it is reasonable to assume that each cell which contains cytochrome P-450 should be able to hydroxylate benzene. The liver seems to be the main organ for benzene metabolism, since partial (70-80%) hepatectomy in rats reduced urinary excretion of benzene metabolites

by 70% (67). Drew and Fouts (68) demonstrated that rat lung microsomes hydroxylated benzene at 10-30% of the rate measured in liver microsomes (these data are in conflict with other results from the same group (59), where rat liver and lung microsomes were stated to have similar specific activities). In addition, they found that the lung activity responded differently to inducers than did the liver activity. Recently, it has been shown that bone marrow metabolizes benzene. This is important since bone marrow is the target organ for benzene toxicity. Andrews et al. (69) demonstrated that rabbit bone marrow microsomes converted benzene to phenol and an additional unidentified metabolite. Irons et al. (38) recently reported that perfused rat bone marrow metabolized benzene to phenol, catechol, hydroquinone and covalently bound metabolites.

A principally very different route for the primary metabolism of benzene than the monooxygenase-mediated hydroxylation was observed in guinea pig liver microsomes by Sloane (70). He demonstrated that benzene was hydroxymethylated to benzyl alcohol via an as yet unidentified mechanism. The significance of this metabolic route is doubtful, since the rate of hydroxymethylation was only 0.1% of the rate of hydroxylation.

Another important aspect of benzene metabolism is the secondary oxidation, i.e. the further oxidative metabolism of phenol. As described above, this matter has been studied in vivo. To the best of my knowledge no in vitro studies have been undertaken except for the very brief one by Harper et al. (59). Considerably more work is required to characterize fully these reactions.

Induction and inhibition of benzene biotransformation

a. Introduction into induction-inhibition of cytochrome P-450-mediated drug metabolism

Cytochrome P-450-mediated reactions can be affected by a large variety of inducers and inhibitors. Two

major classes of inducing agents can be identified:

1. polycyclic aromatic hydrocarbons (the most widely used inducer of this kind is 3-methylcholanthrene) and
2. phenobarbital and a number of compounds which act similarly (71). These two classes of inducers give different patterns of induction as seen by gel electrophoresis and substrate specificity of the induced enzyme species (72, 73). Polychlorinated biphenyls (usually used as the commercially available mixture of isomers, Aroclor 1254) yield a mixed type of induction (74), while safrole and isosafrole seem to induce a type of cytochrome P-450 different from those induced by phenobarbital or 3-methylcholanthrene (75). Several inhibitors have been used to differentiate between the various forms of cytochrome P-450. For instance, metyrapone inhibits the phenobarbital-induced form rather specifically (76) while α -naphthoflavone inhibits the 3-methylcholanthrene-induced form (77). The current concepts of the multiplicity of the cytochrome P-450 system undergo rapid change, and it is not known how many forms of the enzyme are present, or can be induced, in any animal species.

b. Benzene as an inducer of benzene metabolism

Pre-exposure of rabbits, rats and mice to benzene via subcutaneous injections increased the rate of benzene metabolism in vitro about 3-fold (58). Typically, rabbits were given 1.1 benzene/kg body weight on 2 consecutive days and sacrificed on day 3, rats received 2 doses of 1.1 g benzene/kg 18 and 24 h. before sacrifice and mice 1.76 g/kg 12 and 24 h. before sacrifice. If instead 50 mg benzene/kg were given to rats 5 days/week for 1 month, no induction was seen (78). Despite the rather pronounced enhancement of benzene metabolism, no elevation of the cytochrome P-450 content in the liver was observed in mice treated as described above (64), or in rats after 14 daily subcutaneous injections of 1.1 g/kg (79). It was shown, however, that induction by benzene was accompanied by an increased incorporation of radioactive amino acids

into proteins (58), indicating that benzene triggered increased protein synthesis, as do phenobarbital and 3-methylcholanthrene (see below). On the other hand, high doses of benzene given intraperitoneally appear to inhibit in vitro protein synthesis in liver polyribosomes prepared from rats sacrificed shortly (20 min.) after benzene injection (see below).

Norpot et al. (80) exposed Wistar rats to a benzene atmosphere of 450 ppm during 5 h. per day for 10-28 days, and found, contrary to the results cited above, that the concentration of cytochrome P-450 in the livers increased by about 75%. Furthermore, a significant increase in the activity of aminopyrine demethylase was obtained as a result of the benzene exposure. Drew et al. (81) also investigated the effect of benzene inhalation on the metabolism of benzene in rat liver microsomes. At 4,050 ppm benzene for 4 h. per day for 3 days, the rate of hepatic benzene metabolism was about doubled. Exposure to 2,000 or 1,650 ppm for the same length of time gave a slight (about 10%) increase in activity. In none of the cases did benzene inhalation affect the rate of benzene metabolism in rat lung microsomes.

Timbrell and Mitchell (82) tested the effect of benzene pretreatment on urinary excretion of ^{14}C -benzene metabolites after subcutaneous administration of ^{14}C -benzene to rats. During the first 24 h. after ^{14}C -benzene administration there was a 46% increase in metabolites excreted by benzene-pretreated rats compared to untreated ones. During the second 24 h.-period, however, the untreated rats excreted slightly more than the pretreated ones.

The fact that the metabolism of benzene is induced substantially by benzene pretreatment, without the level of cytochrome P-450 being affected, has been taken as an indication that a quantitatively rare form of cytochrome P-450 is responsible for benzene metabolism in untreated animals, and that this form is induced by benzene. However, induction by benzene is not

only demonstrable with benzene as substrate. Aminopyrine (80), zoxazolamine (79), meoprontosil (79), p-nitro benzoic acid (79) and ethoxycoumarin (I) can also be used as substrates to demonstrate the induction.

When benzene is used as an inducer, an increase in hepatic drug metabolism is obtained at a time when no proliferation of the endoplasmic reticulum is seen (79). In this respect, benzene seems to be an inducer with properties more similar to the polycyclic hydrocarbons than to phenobarbital, since following phenobarbital administration, enzyme activities and proliferation of the endoplasmic reticulum appear to be induced at similar rates (83, 84). It must be noticed, however, that induction with benzene does not lead to a blue shift of the absorption maximum of the cytochrome P-450-CO complex (85) as does induction by e.g. 3-methylcholanthrene (86). When benzene was given to rats as daily injections (1.1 g/kg) during 14 days, there was a slight proliferation of the endoplasmic reticulum, but the induction of benzene metabolism was smaller than after only two injections (79).

Snyder et al. (79) also investigated whether benzene metabolites were able to increase the rate of benzene metabolism. Phenol and resorcinol had no effect, while hydroquinone, catechol and hydroxyhydroquinone were inhibitory. Thus, benzene itself, rather than its metabolites, seems to be the inducing agent.

c. Phenobarbital as an inducer of benzene metabolism

Phenobarbital is one of the most widely used inducers of hepatic drug metabolism (71, 83, 84). It also causes many cytological and morphological changes of the hepatic tissue. Phenobarbital treatment is accompanied by proliferation of the endoplasmic reticulum (83, 84), increased incorporation of amino acids into proteins (87), increased activity and synthesis of nucleic acids and related enzymes (88, 89, 90) and increased synthesis of cytochrome P-450 (91).

Many studies have shown clearly that the metabolism of benzene is increased by phenobarbital, both in vivo and in vitro. Snyder et al. (58) compared the effect of phenobarbital and benzene pretreatment on the metabolic conversion of benzene in rabbit liver homogenates. They found that pretreatment with either compound increased the metabolism to the same extent (about 3-fold), but that the effect of benzene was seen at an earlier stage. The stimulating effect of phenobarbital pretreatment on benzene metabolism in subcellular fractions from liver has since been described in rat and hamster (68, 81). In mouse, however, Snyder et al. (64) failed to detect an increase in benzene metabolism despite a 50% increase in the concentration of cytochrome P-450 following phenobarbital administration (as described above, benzene pretreatment of mice increased benzene metabolism without affecting the cytochrome P-450 level). Drew and Fouts (68) demonstrated that phenobarbital pretreatment of rats stimulated benzene metabolism in liver microsomes but not in lung microsomes. On the other hand, chlorpromazine pretreatment increased the activity slightly in the lung but not in the liver. Thus, it is possible that benzene is metabolized by different forms of cytochrome P-450 in liver and lung tissues. This suggestion is supported by the findings of Capdevila et al. (92), who showed the constitutive and 3-methylcholanthrene-induced forms of cytochrome P-450 in rat lung to have distinctly different spectral properties than the forms present in rat liver. Another observation, by Drew et al. (81), is that phenobarbital pretreatment of rats increased the hydroxylation of benzene 3.4-fold in liver microsomes from females and 4.7-fold in microsomes from males.

Several reports demonstrate that the increased metabolism due to phenobarbital treatment also results in an increased urinary excretion of benzene metabolites. However, the effects obtained in vivo are not as pronounced as those in vitro. Ikeda and Ohtsuji (93)

examined urinary metabolites during five 2 h.-periods after benzene administration (0.44 g/kg) to control or phenobarbital-pretreated rats. A significant increase in metabolites was observed only during the first 2 h.-period. Gut (24) demonstrated that phenobarbital pretreatment of rats increased $V_{\max}$ of the hepatic hydroxylation of benzene 6-fold, while K_m remained unaffected. After oral administration of benzene the urinary excretion of metabolites was significantly enhanced in phenobarbital-treated rats compared to control animals only during the first 3 h.-period. The enhancement after intraperitoneal administration was only 26%, a statistically insignificant difference. After subcutaneous administration of benzene there was no difference between control and phenobarbital-pretreated rats in the rate of urinary excretion of metabolites. One explanation offered by the author is that the rate-limiting step in benzene metabolism after subcutaneous injections of benzene is the release of benzene into the blood rather than the metabolic capacity of the liver tissue. In conflict with these results, Timbrell and Mitchell (82) found that phenobarbital as well as benzene pretreatment increased the urinary excretion of ^{14}C -benzene metabolites in rats during the first 24 h. following subcutaneous administration of ^{14}C -benzene. Similarly, Gill et al. (94) found that the urinary excretion of benzene metabolites following subcutaneous administration of benzene to rats increased after phenobarbital and 3-methylcholanthrene pretreatment.

d. 3-Methylcholanthrene as an inducer of benzene metabolism

3-Methylcholanthrene is another extensively used and studied inducer of drug metabolism. It differs from phenobarbital in its inducing properties by not causing a significant proliferation of the endoplasmic reticulum at a time when an increase in enzyme activity is obvious (83). 3-Methylcholanthrene also induces a different form of cytochrome P-450 (cytochrome P-448) with

different substrate specificity (71, 72, 73). An increase in microsomal hydroxylation of benzene is observed in rat liver microsomes but not in rat lung microsomes after 3-methylcholanthrene pretreatment of the animals (68). The lack of effect in the lung is remarkable, since 3-methylcholanthrene induces a specific form of cytochrome P-450 in rat lung (92). Gill et al. (94) demonstrated that 3-methylcholanthrene pretreatment increased the urinary excretion of benzene metabolites in rats who received benzene subcutaneously. The effect of 3-methylcholanthrene was weaker than that of phenobarbital.

e. Other inducers of benzene metabolism

As described above, pretreatment of rats with chlorpromazine slightly enhanced benzene metabolism in lung but not in liver microsomes. Dimethyl sulfoxide (DMSO) pretreatment of rats also enhances benzene metabolism (95). The enhancement is obtained without any increase in the concentration of cytochrome P-450 (96).

f. Inhibition of benzene metabolism

The hydroxylation of benzene is inhibited by a variety of compounds. To examine whether this hydroxylation is a cytochrome P-450-mediated reaction, Gonasun et al. (64) tested the effect of inhibitors of cytochrome P-450 and of other enzymes of potential interest for their effect on benzene hydroxylation. They found that aniline and metyrapone, which produce type II spectral changes in cytochrome P-450, and SKF-525A and aminopyrine, which are type I binding agents, all inhibited the formation of phenol from benzene in mouse liver microsomes, although relatively high concentrations of the inhibitors were required to obtain pronounced effects. For instance, 1 mM metyrapone was necessary for 50% inhibition of the reaction. Gonasun et al. (64) further showed that cytochrome c inhibited benzene metabolism in mouse liver microsomes. This is thought to depend on the ability of cytochrome c to

accept electrons from NADPH-cytochrome P-450 reductase, thus slowing down the cytochrome P-450 reduction. KCN, which is known to inhibit cytochrome oxidase but not cytochrome P-450, was not inhibitory. In addition, CO, a typical cytochrome P-450 inhibitor, also inhibited benzene metabolism.

The catalase inhibitor 3-amino-1,2,4-triazole (97) did not affect benzene metabolism in mouse liver microsomes according to Gonasun et al. (64). However, Nomiyama (98, 99) found that this compound did inhibit benzene metabolism in rats when measured as phenol formation in vivo, or when liver homogenates from rats pretreated with 3-amino-1,2,4-triazole were used.

Various sulphur-containing compounds are known to inhibit biological oxidations. The effect of a series of these compounds - cystine, cystamine, methionine and the synthetic antioxidant propyl gallate - on benzene metabolism was tested in vitro using rat liver homogenates or in vivo in rabbits. There was an inhibition of benzene metabolism by all these compounds in rat liver homogenates. This was also the case in rabbits receiving pretreatment with methionine, or any of the four antioxidants simultaneously with benzene (100).

SKF-525A, a wellknown inhibitor of cytochrome P-450 (71), decreased the urinary excretion of total phenol in rats when administered intraperitoneally 1 h. prior to a subcutaneous injection of benzene (94). Also piperonyl butoxide, which is a cytochrome P-450 inhibitor, and cobalt chloride, which blocks heme biosynthesis and thereby inhibits cytochrome P-450 synthesis, decreased urinary excretion of benzene metabolites in rats during a period of 24 h. after subcutaneous injections of benzene (82). Toluene is another inhibitor of benzene metabolism that has been used widely. Thus, the metabolic formation of phenol from benzene can be substantially inhibited by simultaneous administration of toluene, both in vivo and in vitro in rats and mice. The mechanism appears to be competitive inhibition (33, 101, 102). This is of some

practical importance since there is often a simultaneous exposure to several organic solvents in the chemical industry.

Toxicity of benzene

Some of the most important aspects of benzene toxicity, particularly the hematotoxicity, are discussed in this chapter. The literature on benzene toxicity is very large, and several adverse effects other than those described here have been reported. Thus, both acute and subacute exposures of animals to benzene have resulted in histochemical changes in the spinal chord (103), the endocrine glands (104), the small intestine epithelium (105), the lung (106), the kidney (107) and the liver (108). Benzene is not considered to be a serious hepatotoxic agent although early reports describe centrilobular liver necrosis in animals treated with benzene (109). Centrilobular liver necrosis has also been found in cases of chronic human benzene poisoning (110), but has been interpreted as secondary to hematopoietic injury (109). However, the studies by Jonek et al. (108, 111), showing enzymatic and ultrastructural liver changes, and the study by Wirtschafter and Cronyn (112), reporting signs of cytoplasmic degeneration and leakage of hepatic enzymes into serum after benzene injections, suggest that benzene can act directly as a hepatotoxic agent.

Acute toxicity

Acute benzene toxicity can easily be achieved in animals following exposure via inhalation. About 4,000 ppm will produce narcosis, and exposure to 10,000 ppm is usually fatal within a couple of hours. Rabbits exposed to 35,000-45,000 ppm died after 20-70 min., and death was caused by depression of the central nervous system (113). LD_{50} -values following oral administration of benzene to newly born, 14 days old and adult rats were <1, 3.4 and 5.6 ml/kg body weight, respectively (114).

Subacute and chronic toxicity

a. Toxic effects on hematopoietic tissues (for reviews see refs. 10, 115, 116). It has been known since the beginning of this century that benzene causes damage to the blood-forming organs and changes the differential blood cell counts. Generally, one can differentiate between two major types of adverse effects: 1. cytopenia - aplastic anemia (the term "aplastic anemia" is often used in the benzene literature and means cytopenia plus degeneration of bone marrow tissue) and 2. leukemia.

a.1. Cytopenia - aplastic anemia. The numerous studies of the effects of benzene on the peripheral blood counts and on the bone marrow status have produced confusing and contradictory results. Cytopenia and cytolysis probably occur for all different cell types as a result of benzene exposure. The contradictory results found in the literature are probably due to the time course for the development of adverse effects and to the time course for regeneration processes. Thus, on a single subcutaneous injection of a high dose of benzene (1 ml/kg) to rabbits, a rapid decrease in the number of peripheral leukocytes was observed. However, in 30% of the experimental animals the decrease was preceded by a transient rise in cell numbers (3, 117). In those animals who survived the primary decrease there was a primary rise to about normal leukocyte levels. This rise was followed by a secondary fall, which, in turn, was followed by a secondary rise to the normal level (118). On repeated exposure to high doses of benzene, however, white blood cells will invariably reach very low levels (cf. 118).

In a recent study mice and rats were exposed to a benzene atmosphere of 300 ppm (119, 120). The exposure caused high mortality, lymphocytopenia, anemia, neutrophilia and reticulocytosis in the mice, but only lymphocytopenia in the rats. In another study, it was reported that rats developed moderate leukopenia after exposure for 5-8 weeks to as little as 44 or 47 ppm of

benzene (121). Female rats were slightly more sensitive than male ones. Numerous other reports deal with the cytopenic effects of benzene, and such effects have been demonstrated in rats, mice, rabbits and guinea pigs. For a review of the data, see ref. 10.

Cytopenic effects of benzene have also been demonstrated in humans occupationally exposed to benzene. Aksoy et al. (122) reported abnormal blood values in 51 out of 217 apparently healthy shoemakers, who, during 3 months to 17 years, had been exposed to 30-210 ppm of benzene. Anemia was the most common abnormality, but leukopenia, thrombocytopenia and pancytopenia also occurred. Savilahti (123) investigated 147 workers exposed to about 400 ppm of benzene in a shoe factory. Of these, 107 had abnormal blood counts (most common thrombocytopenia), 10 were hospitalized and one died from severe pancytopenia. Nine years later (1964) 125 of these workers were reinvestigated and compared with a control group (124). The leukocyte levels were restored but the erythrocyte and thrombocyte counts were still lower in those earlier exposed to benzene. The differences between controls and exposed were more pronounced for males than for females in the latter study, while no sex difference could be demonstrated in the original investigation. The degree of recovery seemed to be independent of the severity of the original damage. Goldwater (125) investigated 332 workers in a printing factory who were exposed to 11-1,000 ppm of benzene. Significant cytopenia was noted in 23 cases and lymphocytopenia was the most common abnormality. Wilson (126) studied 1,104 workers in a rubber plant. The exposure to benzene was said to have been up to 500 ppm, averaging 100 ppm. Small hematological abnormalities occurred in 83 cases, 25 had severe pancytopenia, 9 were hospitalized and 3 died.

The effects of benzene on bone marrow have been investigated from many points of view. As illustrated in experiments by Kissling and Speck (127) the effect of benzene on bone marrow may be different in different

individuals. Of 16 rabbits given subcutaneous injections of 0.2 ml benzene per kg body weight/day for 6-12 weeks, 10 showed hypocellular, 4 normocellular and 2 hypercellular bone marrow. Also humans exposed to benzene can exhibit both hypo- and hypercellularity in the bone marrow as shown by Aksoy et al. (122). As treatments continue at high doses for prolonged periods of time, however, the bone marrow gradually degenerates, as shown for instance in mice (V) and rats (128). This bone marrow degeneration together with cytopenia in the peripheral blood leads to aplastic anemia.

Already in 1916 Selling (3) treated rabbits subcutaneously with benzene and carried out histopathological examination of the bone marrow. He found that virtually all cell lines in the marrow were affected by this treatment. When only peripheral blood counts are undertaken, erythrocytes usually appear more resistant to the effects of benzene than the white blood cells. The reason for this is probably that erythrocytes have a considerably longer life-time than most leukocytes. Thus, a disturbance in the erythropoiesis will only slowly show up as a decrease in the number of erythrocytes in blood. Lee et al. (129, 130) have investigated the effect of benzene on incorporation of Fe into reticulocytes. Benzene inhibited this process and this inhibition could be observed even before the number of leukocytes decreased.

Several lines of evidence indicate that the fully mature blood cells and the multipotent stem cells are relatively resistant to the toxic actions of benzene. Targets are, instead, cells undergoing differentiation and proliferation (129, 131, 132). An example of this is the demonstration that committed stem cells, in this case granulopoietic stem cells, are highly sensitive to benzene (133, V, VI).

As mentioned above, benzene is not regarded to be severely toxic to the liver. However, Sammett et al. (67) found that benzene reduced the rate of liver regeneration in partly hepatectomized rats. The tempting

speculation that all proliferating tissues are sensitive to benzene toxicity seems incorrect, however, since other such tissues like skin or intestinal mucosa are not known to be sensitive to chronic exposure to benzene.

a.2. Leukemia. Benzene has for a long time been suspected to cause leukemia in man. The clinical and epidemiological support for this is substantial, and today benzene is generally recognized as a carcinogen in man. However, research on the carcinogenic effects of benzene has been hampered by the lack of a suitable experimental model. All attempts to induce leukemia in animals with benzene have failed (with the exception of one study from 1932 (134), which has been criticized due to the lack of a control group). It is therefore of interest to note a recent report stating that mice exposed to 100 or 300 ppm of benzene during their life-time exhibited a significant increase in hematopoietic neoplasms (119).

The most common form of leukemia in man caused by benzene is acute myelogenous leukemia (13, 14, 135, 136, 137). This syndrome is characterized by neoplastic proliferation of cells closely related to normal myeloblasts. Erythroleukemia and monocytic leukemia may also be induced by benzene exposure (14, 15, 138). Chronic myelogenous leukemia has also been reported to be connected with benzene exposure in a few cases (139, 140). The possible causal relationship between benzene exposure and lymphocytic leukemia (135, 139, 141) appears to be rather tenuous (142).

Leukemia develops only in a small fraction of the cases where hematotoxic effects of benzene can be seen. Out of 147 workers exposed to high (400 ppm) benzene concentrations, 107 had some degree of cytopenia and one developed leukemia (123, 124). The frequency of leukemia has been higher in investigations where only patients seeking medical care are included. In one study (136) 5 cases of leukemia were found out of 41 cases of hematotoxicity. In another study including

83 cases of benzene-induced hematotoxicity, 19 cases of leukemia were found. All leukemia cases and 14 of the other cases of hematotoxicity ended fatally (143). Thus, leukemia caused more deaths than cytopenia and aplastic anemia.

It is not known whether benzene-induced leukemia develops secondary to cytopenia or aplastic anemia, or whether these symptoms are initiated via different mechanisms. In most studies, aplastic anemia has been observed before the development of leukemia. In one study (15, 144), however, it was claimed that leukemia developed in two patients without earlier signs of aplastic anemia. Statements in the literature on latency for benzene-induced leukemia vary considerably; Infante (137) found 10-20 years and Vigliani (14) 3-24 years. Leukemia may also develop several years after cessation of benzene exposure (142).

In most studies no reliable measures of the benzene doses causing leukemia exist. Either have there been no dose measurements at all or has the exposure situation been too complicated to assess the role of benzene. Aksoy et al. (15) stated that 4 workers in a shoe factory developed leukemia after having been exposed to a benzene concentration of 210 ppm during 6, 8, 10 and 12 years. Kinoshita et al. (145) described one case where exposure to 220 ppm for 6 months led to acute myelogenous leukemia. Sellyei et al. (146) reported that exposure to 63-514 ppm benzene for 18 months gave leukemia 7 years after cytopenia. Among 594 rubber workers exposed to benzene concentrations varying from less than 2 ppm to more than 25 ppm, 3 cases of leukemia were observed when only 0.8 were expected (147). The statistical significance is disputed. 748 workers in two rubber factories, who were exposed to benzene during the years 1940-1949, showed a 5-fold higher mortality in leukemia than expected during the years 1950-1977 (137). In one of the factories the exposure was more than 100 ppm during the years 1940-1942 and thereafter 10-15 ppm, and in the

other factory the exposure was up to 500 ppm. Criticism has been raised against this study and the interpretation is not quite clear (148). Other epidemiological studies undertaken in the rubber industry have shown increased frequencies of leukemia and other cancers (149, 150). Since many chemicals and solvents are used in these factories the role of benzene is uncertain. Two recent epidemiological studies on workers in the chemical industry did not reveal any increase in leukemia or significant hematological abnormalities (151, 152). In one of these studies the level of benzene exposure was reported to have been 2-25 ppm, while no exposure levels were reported in the other study.

b. Genetic toxicity. The genotoxic effects of benzene have been documented in numerous experimental studies. Rats inhaling 100 ppm benzene continuously for 4 months had a significantly increased frequency of chromosome aberrations in bone marrow cells. One month after cessation of the exposure, no significant decrease in the number of aberrations was found (153). Kissling and Speck (127) treated rabbits with 0.2 ml benzene/kg body weight/day until the leukocyte counts had fallen below 1,000 cells/mm³. At this stage severe chromosome damage, mainly breaks and gaps, occurred in the bone marrow. The level of aberrant mitosis was 5.9% before treatment and increased to 57.8% after 18 weeks of treatment. More recently, the micronucleus test has proven useful in detecting genotoxicity of benzene. Siou and Conan (154) administered 0.25-5 ml benzene/kg body weight to mice twice orally with 24 h. between the doses. An increased frequency of micronuclei in erythrocyte precursors was observed. Similar results were recently obtained by Hite et al. (155). In another study Lyon (156) found micronuclei in rats following 2 intraperitoneal injections of 0.05 ml benzene/kg body weight.

In several studies benzene has been shown to act as a mitotic poison and to depress synthesis of DNA in cultured human cells (157, 158) and in rabbit and rat

bone marrow cells in vivo (128, 159, 160). The depression of DNA synthesis was observed under circumstances when toxicity was already established. Thus, it is unclear if this effect is a primary or a secondary event.

In cultures of human lymphocytes (activated with phytohemagglutinin) or leukocytes, increased amounts of chromosome aberrations were obtained with 1-2 mM benzene present in the culture medium (157, 158, 161). Morimoto and Wolff (162) found that benzene did not induce sister chromatid exchanges in activated human lymphocyte cultures. On the other hand, phenol and, particularly, catechol and hydroquinone were active. Diaz et al. (163) found that benzene inhibited sister chromatid exchanges in human lymphocyte cultures activated with phytohemagglutinin but increased this process in non-activated cells. This indicates that benzene, or its metabolites, has different effects at different stages of the cell cycle. Diaz et al. (163) also found that the presence of rat liver microsomes in the culture medium further increased the frequency of benzene-induced sister chromatid exchanges in non-activated lymphocyte cultures, indicating that metabolites of benzene rather than benzene itself were responsible for these effects. Tice et al. (164) recently reported that exposure of mice to a benzene atmosphere of 3,100 ppm for 4 h. significantly increased the frequency of sister chromatid exchanges in bone marrow cells. Benzene is non-mutagenic in the Ames' test (156, 165).

There is ample evidence that benzene can induce both stable and unstable chromosome aberrations in humans occupationally exposed to benzene. Although the significance of low frequencies of chromosome damage is uncertain, these effects have to be taken seriously, since there is strong evidence that some kinds of leukemia are related to specific chromosome damage (166). Furthermore, it has been claimed that stable chromosome aberrations, induced by benzene exposure, transformed cells to leukomogenic clones in humans (167, 168).

Significantly increased levels of chromosome damage can remain several years after cessation of benzene exposure. The unstable aberrations tend to disappear, but the stable ones persist and even increase with time (169). Chromosome aberrations in man seem to evolve at rather low levels of benzene exposure. Thus, an increase in chromosome damage in leukocytes was demonstrated in workers exposed to less than 25 ppm of benzene (170). 12 workers, exposed to 5-10 ppm during benzene distillation in a coke plant, exhibited increased frequency of chromosome and chromatid breaks as compared to a control group (18; 171). Picciano (19) investigated 52 workers in a rubber factory exposed to less than 10 ppm benzene and found an increase in chromatid breaks when compared to an unexposed group of 44 individuals. These data have to be interpreted with some precaution, however, since the workers probably were exposed to several potentially harmful compounds besides benzene. In addition, smoking alone has been shown to increase chromosome damage (171), and this factor was not considered in the study by Picciano.

c. Teratogenic and embryotoxic effects. Gofmekler (172) investigated the embryonic development in rats exposed to benzene concentrations varying from 0.3-200 ppm during 10-15 days before impregnation. The number of the offspring was inversely related to the exposure down to 20 ppm, and animals with the highest exposure did not at all become pregnant. It was also noted that the ratio of organ (liver, lung, spleen and kidney) weight:body weight was significantly higher in rats exposed to 20 ppm than in controls. In other studies mice and rabbits were exposed to up to 500 ppm benzene during gestation. No adverse effects were seen in rabbits or their offspring. The fetal weights of mice were decreased at 500 ppm, and some effects on the ossification processes were observed in the mouse offspring (173). Rats exposed to benzene concentrations of 100, 300 or 2,200 ppm 6 h./day during days 6-15 of

gestation gained less weight at the highest exposure level than a control group. In addition, the fetuses of the rats exposed to 2,200 ppm weighed less than those of unexposed animals. The ossification process in the fetuses was affected at the two highest levels of exposure (174).

Mice were treated subcutaneously with benzene (3 ml/kg body weight) during days 11-15 of gestation. Those treated on day 13 showed an increased frequency of malformed fetuses (175). In another study mice were given 0.3-1.0 ml benzene/kg body weight during days 6-15 of gestation. Maternal lethality and embryonic resorption increased at 0.5 and 1.0 ml/kg. The fetal weights were significantly reduced even at the lowest dose. No malformation of fetuses was found (176).

A woman with severe benzene-induced cytopenia in the eighth month of pregnancy was delivered of a healthy boy. Blood samples from the mother showed an increased level of chromosome aberrations but a cytogenetic study of the boy revealed no abnormalities (169).

Thus, experimental data show that benzene exposure may be teratogenic and embryotoxic in animals. The available data are insufficient for an evaluation of whether realistic doses of benzene can cause similar effects in humans.

d. Toxic effects on the immune system. Since bone marrow is both the main target organ for benzene toxicity and an important organ for the immune defence, it is not surprising that benzene exposure can affect the immune system.

Rabbits repeatedly exposed to benzene showed increased susceptibility to tuberculosis (177) and pneumonia (178). Furthermore, rabbits injected subcutaneously with 1 ml benzene/kg body weight had a decreased capacity to produce antibodies against sheep erythrocytes (179). Rabbits treated in the same way with 0.25-0.5 ml benzene/kg body weight/day had a severely reduced number of B-lymphocytes. T-lymphocytes were also reduced but not as markedly. In addition, increased

levels of IgM and lowered levels of IgG were noted (180).

Lange et al. (181, 182, 183) investigated the effects on the immune system of painters exposed to benzene (5-42 ppm), toluene (41-63 ppm) and xylene (66-86 ppm). Levels of IgA and IgG were lower and IgM was higher than in a control group. A significant decrease in the level of serum complement activity was also noted.

Relationship between benzene metabolism and toxicity

The toxic effects of many chemically relatively inert compounds are believed to develop only after metabolic activation. This means that metabolites rather than the parent substances are the toxic species. The metabolites thought to be ultimate toxic agents usually have electrophilic properties and thus possess the ability to bind covalently to nucleophilic sites in cellular macromolecules. Thereby the cellular processes can be disturbed and mutation, cancer or other types of cytotoxic effects may develop (184-190).

The metabolic activation of several compounds have been studied in great detail. The ultimate toxic metabolites can probably be of several types, e.g. primary or secondary arene oxides, conjugates with the ability of easily forming organic ions, quinones, or radicals like semiquinones (184-190).

Benzene is among the compounds believed to require metabolic activation to exert its toxic effects, although the evidence for this is not firm enough to exclude all doubts. Ikeda and Ohtsuji (93) demonstrated that pretreatment of rats with phenobarbital protected the animals against the leukopenic effects of benzene. This finding was later confirmed by Mitchell (191), Drew et al. (81) and Cill et al. (94). Since phenobarbital induces the metabolism of benzene, these findings were interpreted to indicate that benzene itself rather than benzene metabolites was the toxic agent (93). The

general opinion today, however, is that this conclusion, though apparently logical, is incorrect. The enhancing effects of phenobarbital on drug metabolism seem to be mainly restricted to the liver (71). Hence, it is possible that an increased metabolism in the liver will remove more benzene from the circulation and thereby reduce the amount of metabolites formed in the marrow, or hasten the removal of a toxic metabolite. On the other hand, Tice et al. (164) recently reported that the genotoxic effects (sister chromatid exchanges and chromosomal aberrations) in bone marrow caused by benzene inhalation were more severe in rats pretreated with phenobarbital than in untreated animals. The other most frequently used compound to increase drug metabolism, 3-methylcholanthrene, increased the metabolism of benzene but did not affect the degree of leukopenia (94). Snyder (53) investigated the benzene-induced suppression of Fe uptake into reticulocytes in mice and found that those animals with the most effective benzene metabolism also were more susceptible to benzene toxicity. Other reports have shown that inhibitors of benzene metabolism also will protect against benzene toxicity. This has been shown for toluene (33), piperonyl butoxide (191), cobaltous chloride (192) and aminotriazole (98, 99, 192). SKF-525A, a commonly used cytochrome P-450 inhibitor, had no effect on benzene hematotoxicity (94). In summary, the most reasonable interpretation of the induction-inhibition data cited above would be that metabolic activation is a prerequisite for development of the toxic effects of benzene.

There have also been attempts to test several benzene metabolites for their potential as hemotoxic agents in animals. Nomiyama (193) found that of the investigated compounds phenol, catechol, hydroquinone, hydroxyhydroquinone, trans-trans-muconic acid, phenyl mercapturic acid and phenyl sulphate, only catechol appeared to depress bone marrow function in rats by causing anemia and leukopenia. Benzene itself had severe such effects. However, Mitchell (191) could not

reproduce these results; catechol and hydroquinone, at doses lethal to 50% of the rats, did not produce hemotoxic effects (blood counts, bone marrow cellularity). When interpreting experiments where metabolites have been administered in vivo, one thing has to be kept in mind: it has never been examined whether a substantial amount of these metabolites will reach the bone marrow. It is possible that a major fraction will be conjugated in the liver or in other organs and, therefore, never reach the critical organ.

The effects of benzene and its metabolites on cell cultures have also been tested. The advantage when using cell cultures is that all problems with distribution of metabolites in the organism are circumvented. Already in 1948 Harrison and Randoll (194) reported that benzene did not show any significant cytotoxicity in bone marrow cultures derived from rabbits, guinea pigs or rats, while phenol and trihydroxybenzenes were moderately toxic, and the dihydroxybenzenes (catechol, hydroquinone and resorcinol) were very toxic. Similarly, Mitchell (191) reported that only very high benzene concentrations (>6 mM) were toxic to bone marrow cultures. The techniques for culturing bone marrow cells have developed rapidly, however, and only recently have methods been established that allow multipotent stem cells to be produced and continuously committed in culture during time periods of weeks (195). As already mentioned, human lymphocyte cultures were highly sensitive to hydroquinone and, particularly, to catechol when cell division kinetics and sister chromatid exchanges were measured, while benzene had no effect and phenol only a slight effect (162). Thus, the cell culture work indicates that dihydroxybenzenes, or products of these, are the toxic agents.

Benzene oxide is another possible toxic metabolite of benzene. Due to the instability of this compound very little work has been performed to test its effects. Very recently, benzene oxide was shown to be mutagenic in microbiological tests (196). The compound, or its

decomposition product phenol, was also very cytotoxic. Thus, at the highest dose tested (20 mM) the number of revertants/ 10^6 cells was more than 10-fold higher than the control level, but only 0.2% of the cells had survived

Another much debated question is where the metabolism producing the metabolites toxic to bone marrow takes place. Most of the metabolism of benzene occurs in the liver, but as described earlier the bone marrow also possesses the ability to metabolize benzene. Therefore, the two most likely alternatives are that the toxic metabolites either are formed in the liver and transported to the bone marrow, or that they are formed within the bone marrow. Indications in the literature point in both directions. Mitchell (191) and Sammett et al. (67) showed that partial hepatectomy in rats alleviated the bone marrow toxicity of benzene, indicating that the amount of liver metabolites was crucial. Another piece of evidence pointing in the same direction is the mild toxic effect of benzene, as compared to the effects of catechol and hydroquinone, on bone marrow cultures, indicating that the metabolism in the bone marrow cells is not sufficient to produce toxicity (194). On the other hand, Irons et al. (38) showed that the bone marrow does have the ability to form these metabolites since phenol, hydroquinone, catechol and covalent binding to tissue constituents could be detected after administration of benzene to perfused rat bone marrow. Furthermore, the benzene metabolites phenol, hydroquinone and catechol have been reported not to reproduce the severe hemotoxic effects of benzene when administered in vivo (191, 193). If these compounds are formed in the liver and then transported to the bone marrow to exert their toxic effects, they should also be at least as toxic as benzene when administered in vivo. One possibility is that benzene oxide is the toxic agent formed in the liver and then transported to the bone marrow. It seems highly unlikely, however, that appreciable amounts of

this unstable compound would survive such a transport. Nevertheless, it would be very interesting to investigate the effects of benzene oxide when administered in vivo.

Thus, although there is a general agreement that benzene requires metabolic activation to exert its toxic effect on the bone marrow, it is still uncertain which is the most important active metabolite or where this metabolite is formed.

Molecular site of benzene toxicity

As described above, it is generally assumed that the toxic effects of benzene are exerted by some reactive metabolite(s) which interact covalently with cellular macromolecules. The possible effects on the molecular level of such interactions are of several kinds. It is not known which are the primary or most important ones.

As described, benzene metabolites can bind to DNA in vivo, and it is likely that such DNA-binding or binding to the spindle apparatus is the mechanism underlying the genotoxic effects of benzene. It has also been observed that benzene can act as a mitotic poison and inhibit nucleic acid synthesis (128, 157-160). The decrease in nucleic acid synthesis was observed at a stage when benzene toxicity was severe, and it is, therefore, uncertain whether the effect was primary or secondary. In a recent report, Irons et al. (131) could not demonstrate any decrease in DNA-synthesis in bone marrow cells after a benzene dose producing moderate lymphocytopenia. Benzene, phenol and catechol also appear to inhibit DNA-repair. Thus, a slower repair of UV-damaged DNA in the presence of these compounds was observed in cultures of human lymphocytes (197, 198).

Another toxic property of benzene described above is its inhibitory effect on the incorporation of Fe into erythrocyte precursors. It is not known if this

is due to inhibition of heme biosynthesis (see below), or if there is some other effect on erythropoiesis.

Protein synthesis is also affected by benzene. This was shown by Tryfiates (199) and Tryfiates and Choulis (200) who studied the influence of benzene on the translation processes in rat liver. Single intraperitoneally injected doses of benzene ranging between 0.5-5.5 mmoles/100 g body weight caused disaggregation of polyribosomes and accumulation of ribosomal monomers and dimers. Disaggregated polyribosomes from benzene-treated rats had a considerably impaired capacity for protein synthesis. The incorporation of RNA-precursors into polyribosomes was decreased by the benzene treatment, but the incorporation into total cell RNA was normal. These results suggest an effect of benzene on the initiation of protein synthesis.

Freedman et al. (201-203) have studied the effects of benzene on heme and protein biosynthesis in human and rabbit reticulocytes. Protein synthesis in this and several other biological systems is regulated by a "hemin controlled repressor" which will block protein synthesis in the absence of hemin. It was demonstrated that benzene inhibited protein synthesis. The mechanism for this was probably through inhibition of heme synthesis, since addition of hemin to the system reversed the inhibitory effect of benzene. The inhibition of heme synthesis probably occurred before the d-aminolevulinic acid step, since addition of this acid also reversed the effects of benzene. Studies by Kahn and Muzyka (204) are in conflict as to the level of benzene inhibition of heme biosynthesis. They found d-aminolevulinic acid to accumulate during benzene poisoning, and suggested that the inhibition took place at the level of incorporation of Fe into protoporphyrin.

Thus, benzene interferes with several processes on the molecular level. It is not known which is the primary event, or which is the most important one for development of adverse effects. It might well be that more than one mechanism have toxicological relevance.

It is possible, for instance, that covalent binding to DNA is a major primary event for development of genotoxicity and leukemia, while inhibition of protein and heme synthesis might be important for the occurrence of other cytotoxic effects.

THE PRESENT STUDY

Aims

As reviewed above, there is general agreement that benzene requires metabolic activation to exert its toxic effects, but it is not known which is (are) the ultimate toxic metabolite(s). When this study was initiated, knowledge of benzene biotransformation in isolated microsomes was surprisingly poor. The only metabolites known to be formed were phenol (57-59, 64) and benzyl alcohol (70), and no attempts had been made to study covalent binding to macromolecules in vitro. Thus, our main objectives when starting these studies were to answer the following questions: 1. Do metabolites bind covalently to macromolecules during microsomal metabolism of benzene? 2. If binding occurs, which is (are) the main binding agent(s)? and 3. Does the binding to microsomal macromolecules occur randomly or specifically? Results of these studies on the metabolic activation of benzene are presented in papers II, III and IV.

During the studies of the metabolic activation of benzene, we noticed some unusual properties of the benzene monooxygenase in rat liver microsomes. We therefore investigated this enzyme and compared some characteristics of the constitutive and the phenobarbital- and benzene-induced forms. Results of this study are presented in paper I.

All in vitro experiments described in papers I-IV were undertaken with microsomes from rat liver as biological material. As has been described above, there are conflicting reports as to how liver metabolism contributes to the development of the adverse effects of benzene in bone marrow. Whatever the role of liver metabolism for toxicity might be, we considered it to be of basic importance to investigate the biotransformation of benzene in liver microsomes in detail, with particular emphasis on metabolic activation. It is rather surprising that no such studies had been undertaken much earlier with this the simplest of all aromatic hydrocarbons, which, in addition, is of great toxicological interest, while an immense literature exists on metabolic activation of polycyclic aromatic hydrocarbons.

In the second part of this study, papers V and VI, we investigated effects of benzene and some of its metabolites on bone marrow in mice. The parameters studied were: 1. Cellularity. 2. Absolute amount and frequency of colony forming granulopoietic stem cells (CFU-C), and 3. Occurrence of micronuclei in erythropoietic cells. The aim of this part of the study was first of all to see if the results on the metabolic activation of benzene, obtained with rat liver microsomes, were relevant when studying the toxicity in the target organ. Another thing we kept in mind during these studies was the possibility of differentiating between different effects on the bone marrow. As discussed in the review, benzene has been indicated to affect virtually all cell lines in the bone marrow, and the effects on the molecular level are of several kinds. Of the parameters studied, one measures the overall cellularity of the marrow, the second (CFU-C/tibia and CFU-C/ 10^5 cells) selectively measures effects on granulopoiesis, and the third (micronucleus test) measures genotoxic effects on the erythropoietic cell line. It might be that different adverse effects are caused by different metabolites or by benzene itself.

Thus, it was of interest to investigate the degree of adverse effects of benzene on the different parameters as compared to the possible effects of its metabolites.

Results and discussion

a. Benzene monooxygenase

Studies by Gonasun et al. (64) indicated that benzene is hydroxylated by a cytochrome P-450-dependent monooxygenase in mouse liver microsomes. Rat liver microsomes were considerably less active in the hydroxylation of benzene; the specific activities for mouse and rat were 31 and 3.5 nmoles phenol formed/mg microsomal protein/h., respectively. Gonasun et al. (64) further claimed that maximal rate of benzene metabolism was obtained at a benzene concentration of 1-2 mM in mouse liver microsomes, and at 2-4 mM in microsomes from rat liver. Harper et al. (59) used as high concentrations as 11.2 mM (the solubility limit for benzene in water) in their incubations. They obtained a $V_{\max}$ for benzene hydroxylation in rat liver microsomes of 1.35 nmoles phenol formed/mg microsomal protein/min., and the K_m was 4.86 mM. At an initial benzene concentration of 10 mM, 0.02% of the benzene was recovered as phenol after incubation at 37°C for 30 min.

In our in vitro studies we did not intend to perform any detailed investigations on the enzyme kinetics. Instead, our primary interest was to detect possible minor and/or secondary metabolites. To achieve this, the specific activity of the benzene had to be as high as possible, and, for economical reasons, we used low benzene concentrations. In papers I, II and III we used a benzene concentration of 7 μ M (in IV 18 μ M) and 1-1.5% of the benzene was recovered as phenol after incubation for 30 min. under standard conditions. It should be kept in mind that when examining toxicological aspects, it is not appropriate to consider only the concentration necessary for maximal activity of the enzymes. More important is to work with concentrations

that could arise in a reasonable in vivo situation. Rickert et al. (35) showed that the steady state concentration of benzene in blood in rats exposed to a benzene atmosphere of 500 ppm was about 150 μ M (the steady state concentration in bone marrow was around 500 μ M). Further, Andrews et al. (33) found that the benzene level obtained in blood was similar to that observed in the liver of mice exposed to benzene via subcutaneous injections. Thus, at benzene exposure levels encountered today in factories with potential exposure to benzene (up to 10 ppm), the concentration of benzene in the liver will most likely be much below 10 μ M.

During our studies of the metabolic activation of benzene in rat liver microsomes, we tried to trap intermediary phenol by adding UDP-glucuronic acid (the cofactor of UDP-glucuronosyl transferase) to the incubation mixtures (II). The UDP-glucuronosyl transferase is located in the endoplasmic reticulum and thus present in microsomes. Its activity is greatly enhanced by the addition of detergents which presumably act by partly abolishing a permeability barrier (205). Thus, we tried to trap the phenol more effectively by adding the detergent Renex 690 to the incubation mixtures. Quite unexpectedly, the amount of phenol recovered in the presence of the detergent was much higher than in standard incubations. The effect of the detergent on the hydroxylation of benzene was further investigated (I). For comparison, 7-ethoxycoumarin, a widely used substrate for cytochrome P-450, was included in the studies. Using liver microsomes from untreated rats, we found that a maximal stimulation (about 3-fold) of benzene hydroxylation by detergent was obtained at a ratio of detergent to microsomal protein of around 0.5 (w/w). The ethoxycoumarin de-ethylase was inhibited at all detergent:protein ratios.

Microsomes prepared from benzene-pretreated rats hydroxylated benzene 3 times faster than microsomes from untreated rats. The benzene-induced activity re-

sponded to addition of detergent in the same way as did the control activity, i.e. at a detergent:protein ratio of 0.5, 3 times more phenol was recovered than after incubation without the detergent. Also when ethoxycoumarin de-ethylase was measured, benzene pretreatment enhanced the activity about 3-fold. Renex 690 inhibited this activity in microsomes from both untreated and benzene-pretreated rats by 30-40%. Phenobarbital-pretreatment of the rats also increased the rate of benzene hydroxylation and ethoxycoumarin de-ethylase in liver microsomes about 3-fold. The phenobarbital-induced activity, however, responded differently to the presence of detergent than did the control and benzene-induced activity. The rate of the enzymatic reactions in the presence of Renex 690 was about the same in microsomes from phenobarbital-treated rats as in control microsomes, i.e. it appeared as if the activity induced by phenobarbital was almost completely abolished by the detergent.

The commonly used cytochrome P-450 inhibitor metyrapone was also used to compare benzene hydroxylase and ethoxycoumarin de-ethylase in liver microsomes from untreated and treated rats. Also to this modulator, the control and the benzene-induced activities responded similarly while the phenobarbital-induced activity responded differently. For instance, at moderate metyrapone concentrations the benzene hydroxylation was stimulated in microsomes from control and benzene-pretreated animals, while it was inhibited in microsomes from phenobarbital-pretreated ones. Activation of cytochrome P-450-mediated reactions by metyrapone has been described for actanilide hydroxylation (206, 207).

Based on the findings in I, the following conclusions about the nature of the constitutive and induced benzene monooxygenases can be drawn: 1. An unknown number of monooxygenase forms exist in liver microsomes from untreated rats which accept benzene as substrate and some of these are activated by metyrapone and the detergent Renex 690. 2. Phenobarbital induces

monooxygenases which also accept benzene as substrate. These are basically different from the constitutive benzene monooxygenase(s) in that they are inhibited by metyrapone and detergent. 3. Benzene pretreatment induces benzene monooxygenase(s) with properties much resembling, or possibly identical to, those of the enzyme forms present in microsomes from untreated rats.

b. Metabolic activation of benzene

Although data in the literature are somewhat conflicting, there is an agreement that benzene requires metabolic activation to exert its toxic effects (see the review above for a detailed discussion). It has been reported that benzene metabolites will bind covalently to liver DNA in rats inhaling benzene (36), and that several organs of mice will contain irreversibly bound benzene metabolites after subcutaneous administration of benzene (37). The experiments described in II and III were designed to investigate the mechanism of metabolic activation of benzene in rat liver microsomes.

When ^{14}C -benzene was incubated with rat liver microsomes in the presence of a NADPH-generating system, ^{14}C -phenol was the only metabolite which could be identified. However, metabolites were also found to bind irreversibly, presumably covalently, to microsomal macromolecules. The covalently bound fraction after incubation for 1 h. was as high as 20% of the total amount of metabolites. The properties of this covalent binding were studied, and the following observations were made: 1. A NADPH-generating system was required for binding. 2. The binding was reduced by the presence of 2 mM reduced glutathione or 2 mM cysteine (indicating a spontaneous chemical reaction of the reactive metabolite with the SH-group, since cysteine is not a substrate for the glutathione transferases). The decrease in binding was compensated for by occurrence of water soluble metabolites. 3. Protease treatment of the incubation mixture after incubation strongly reduced the recovery-of irreversibly bound metabolites

while RNase treatment had little effect, indicating that binding occurred preferentially to proteins and not to RNA (II).

The aim of our further research was to identify the benzene metabolite responsible for the covalent binding. We, and probably all other researchers in the field, were convinced at this time that the binding agent was benzene oxide, and that the metabolic activation of benzene was as depicted in Fig. 2. This conviction was based primarily on findings for polycyclic aromatic hydrocarbons, where primary or secondary epoxides, had been indicated to be binding agents (for reviews see refs. 184-190). Further, Jerina et al. (65) had shown that metabolism of benzene oxide in

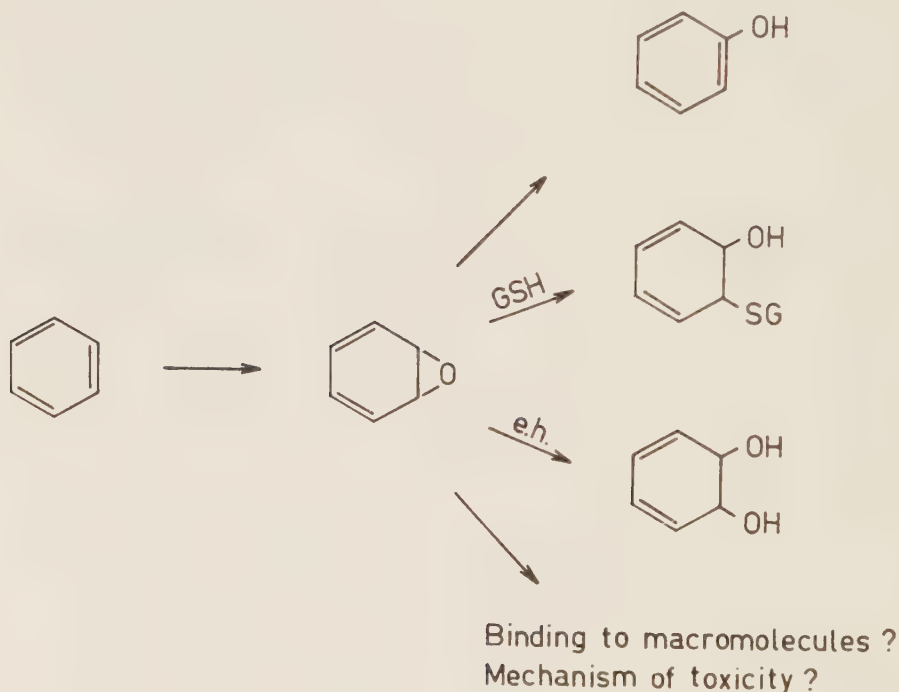


Fig. 2. Generally accepted pathway for the metabolic activation of benzene at the time when our studies were started (e.h., epoxide hydratase; GSH, reduced glutathione).

vitro yielded the same main groups of metabolites as did the metabolism of benzene in vivo. Attempts by these researchers to isolate metabolically formed benzene oxide were unsuccessful. In addition, benzene dihydrodiol was reported to be present in traces in urine of rabbits after a heavy dose of benzene (50). Since this latter study was performed in vivo, a contribution to benzene metabolism from intestinal bacteria cannot be excluded. Multi-step reactions leading to dihydrodiol formation via routes not including an epoxide might therefore be possible. As indicated above, we could not demonstrate benzene dihydrodiol as a metabolite of benzene after incubation with microsomes only. However, the diol could be detected as a metabolite of benzene when large amounts of purified epoxide hydratase were added to the incubations (II). This was the first demonstration of metabolic formation of benzene dihydrodiol from benzene in vitro. The formation of the diol on addition of epoxide hydratase to this simple biological system represents an unequivocal proof for the metabolic formation of an intermediate benzene oxide, which has so far eluded isolation.

Further experiments demonstrated, to our surprise, that benzene oxide could not be the main binding benzene metabolite in our system. The most direct piece of evidence was that tritiated benzene oxide did not bind covalently to an appreciable extent when added to a microsomal incubation. If glutathione was present, the conjugation of benzene oxide to glutathione was negligible (II). This was in contrast to incubations with benzene. Then 20% of the metabolites bound covalently, and this binding was almost completely inhibited by the presence of glutathione, leading instead to water soluble compounds. Another piece of evidence also indicating that benzene oxide was not the binding agent was the fact that the presence of 5 mM reduced glutathione in the incubation mixtures, when ^{14}C -benzene was substrate, inhibited the covalent binding by about 95% but the phenol formation by less than 20%

(II). This would be difficult to explain if benzene oxide were the immediate precursor both for phenol and covalent binding.

Benzene oxide being excluded as the main binding benzene metabolite, we examined the possibility that newly formed phenol could be further metabolized to reactive species. Actually, when tritiated phenol was incubated with rat liver microsomes and a NADPH-generating system, we could demonstrate a several-fold higher level of covalent binding to macromolecules than with benzene as a substrate (II). Other experiments indicated that phenol was an intermediate in the formation of binding metabolites when ^{14}C -benzene was substrate; the presence of UDP-glucuronic acid in the incubation mixtures inhibited covalent binding by up to 80%, radioactivity accumulating instead in the water phase. Since phenol is an excellent substrate for microsomal UDP-glucuronosyl transferase (208) water soluble conjugates are expected to be formed with phenol in the presence of UDP-glucuronic acid, diverting the phenol from further oxidative metabolism and binding. The level of benzene oxide, on the other hand, should not be affected by the presence of this cofactor.

Because of the observations presented above the possible pathways for the metabolic activation of phenol became of interest. The oxidative metabolism of phenol has not been investigated in vitro. For instance, it is not known if the hydroxylation of phenol proceeds via an epoxide intermediate. Results obtained in vivo indicate that hydroquinone is the major further hydroxylated metabolite of phenol, while catechol, if formed at all from phenol, is a minor product (46, 47). As a metabolite of benzene catechol can be formed via dehydrogenation of benzene dihydrodiol (48). Aromatic dihydroxylated hydrocarbons can be oxidized to semiquinones and quinones. For instance, α -methyldopa (209), 2-hydroxyestrone (210) and ethynyl estradiol (211) are most likely converted to covalently binding

semiquinones and/or quinones by microsomes. The oxidizing agent forming semiquinones from hydroquinones is probably the superoxide radical (209, 212), although an oxidizing effect of molecular oxygen cannot be excluded. Superoxide dismutase is an enzyme which catalyzes the dismutation of superoxide radicals (213). Therefore, we tested the effect of superoxide dismutase on the covalent binding to macromolecules in microsomal incubations where ^{14}C -benzene or ^{14}C -phenol were substrates (III). The covalent binding of both benzene and phenol metabolites was reduced by more than 50% by superoxide dismutase, and instead there was an accumulation of ^{14}C -hydroquinone. These results show hydroquinone to be a metabolite of benzene and phenol in vitro, but, in the absence of superoxide dismutase, it is apparently almost quantitatively converted to covalently binding metabolites (probably p-benzosemiquinone and/or p-benzoquinone). The effect of superoxide dismutase also suggests that the superoxide radical is the most important oxidizing agent in this activation process. Since the dismutase reduced the binding by more than 50%, the semiquinone and/or the quinone seem to be quantitatively important reactive metabolites of benzene.

Another possibility to get insight into the mechanism of metabolic activation of benzene was to try to determine the structure of the glutathione conjugate formed during metabolism. In order to obtain a reference substance a conjugate was prepared chemically from p-benzoquinone and reduced glutathione. This conjugate was identified by field desorption mass spectrometry as 2-(S-glutathionyl) hydroquinone. Incubations of microsomes and a NADPH-generating system with benzene, phenol, hydroquinone or p-benzoquinone in the presence of reduced glutathione gave rise to a peak eluting from the high pressure liquid chromatograph at a retention time identical to that of the chemically prepared 2-(S-glutathionyl) hydroquinone. The microsomal metabolite of p-benzoquinone, hydroquinone and phenol thus

eluting from the high pressure liquid chromatograph was further identified as 2-(S-glutathionyl) hydroquinone by field desorption mass spectrometry (III). The glutathione adduct formed from benzene eluted from HPLC with the same retention time and its mass spectrum also contained the molecular ion of this conjugate as an intense peak. The fragmentation patterns did not allow definitive assignments, however, probably due to the small amounts of ultimate reactive metabolites formed from this pre-precursor and the resulting relatively large amounts of impurities.

The results obtained in these studies show, as depicted in the upper part of Fig. 3, that the metabolic

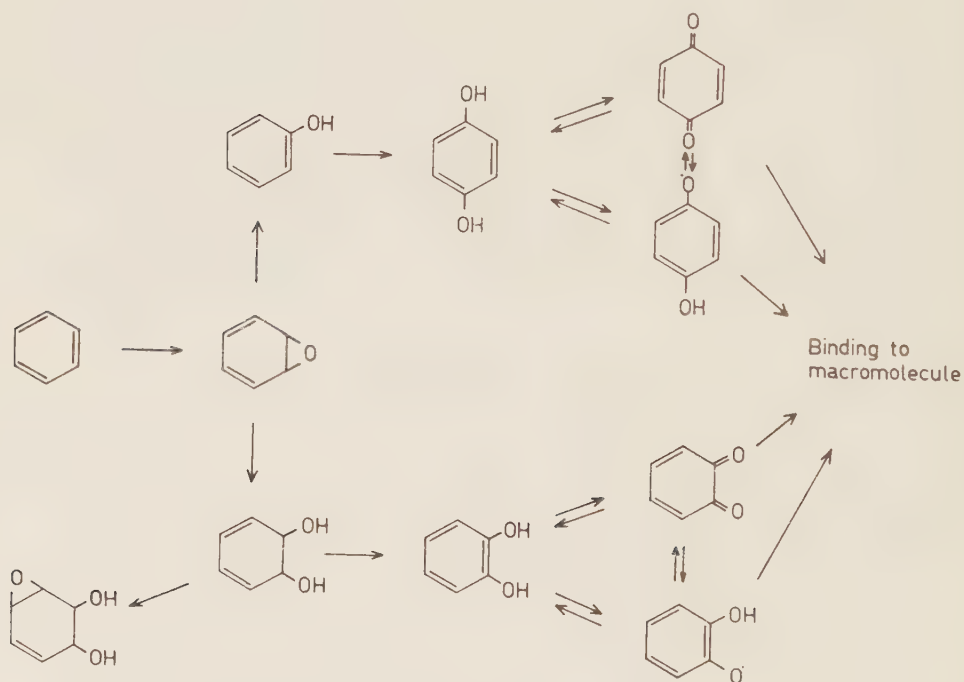


Fig. 3. Pathways for the metabolic activation of benzene. The upper part of the figure depicts the results of this study, while the lower part (starting with hydration of benzene oxide) shows a hypothetical pathway for metabolic activation that might be significant in vivo.

activation of benzene in rat liver microsomes is a multi-step process involving benzene oxide, phenol and hydroquinone as intermediates and p-benzosemiquinone and/or p-benzoquinone as the major covalently binding metabolites. In the lower part of Fig. 3 a hypothetical alternative pathway for metabolic activation of benzene is shown, including benzene dihydrodiol-epoxide, o-benzosemiquinone and o-benzoquinone as reactive metabolites. No evidence for the occurrence of these metabolites could be obtained in our studies, probably because the dihydrodiol was a minor metabolite of benzene and because formation of catechol from the dihydrodiol is dependent on a cytosolic enzyme (48). The possibility remains, however, that this route of metabolic activation is significant in vivo, particularly since catechol in most studies in vivo has been identified as a metabolite of benzene (see the chapter "Biotransformation of benzene: In vivo").

c. Covalent binding to specific microsomal proteins

It is difficult to estimate the toxicological importance of the irreversible, presumably covalent, binding of xenobiotics to microsomal macromolecules during their metabolism in vitro. The clearest indication that such binding can be of toxicological significance was obtained during investigations of the mechanism of action of the hepatotoxic agent bromobenzene (for a review see ref. 185). Tissue necrosis and massive covalent binding occurred only after depletion of glutathione. This indicated a causal relationship between covalent binding and toxicity.

The basic idea of our study on binding specificity was that development of toxic effects might be dependent more on interaction with particular target macromolecules than on the overall amount of binding. This concept is accepted to be relevant for binding of at least some carcinogens to DNA. In that case, the quantitatively minor binding to the O-6-site in guanosine of some carcinogens correlates much better with the

tumor incidence than does the overall binding to DNA. The reason for this is probably that this interaction will affect the selectivity of the base pairing process (for an overview, see ref. 186).

The studies presented in IV were carried out to answer the following basic questions: 1. Do toxic and/or carcinogenic hydrocarbons bind specifically or randomly to microsomal proteins during microsomal metabolism? 2. If there is a specificity, do different compounds have different binding patterns?

Radiolabelled hydrocarbons were incubated with microsomes and a NADPH-generating system. After incubation, the proteins were separated by SDS-polyacrylamide gel electrophoresis and the binding patterns investigated by fluorography. Benzene, phenol and chlorobenzene gave very similar binding patterns. The most striking feature was the dominance of a radioactive band with a molecular weight of about 72,000. The other two hydrocarbons studied, benzo(a)pyrene and 3-methylcholanthrene, gave binding patterns similar but not identical to each other, but distinctly different from those of benzene, phenol and chlorobenzene.

These results are interesting to consider from the point of view of metabolic activation. As shown in II and III, p-benzosemiquinone and p-benzoquinone are important reactive metabolites of benzene and phenol, while many reports indicate that different types of epoxides are the main binding agents of benzo(a)pyrene and 3-methylcholanthrene (188, 214-216). Thus, it is possible that different pathways of metabolic activation are reflected in different binding patterns. The underlying mechanism for the binding specificity may be of two kinds: 1. Each type of reactive metabolite may select target molecules with particular affinity for this type of metabolite. 2. The reactive metabolites may bind close to their site of formation. The fact that chlorobenzene gave the same binding pattern as did benzene and phenol indicates that quinones and/or semiquinones are important binding metabolites of

this compound. Recent results (Tunek and Oesch, submitted for publication; Hesse and Wolff, poster presented at the 2nd International Symposium on Biological Reactive Intermediates, Guildford, U.K., 1980) do indicate that bromophenol is an intermediate in the metabolic activation of bromobenzene by microsomes, and that bromobenzene-3,4-epoxide contributes only with a minor part of the covalent binding to microsomal macromolecules.

The basic findings presented in IV show that toxic and/or carcinogenic substances bind specifically and differently to microsomal proteins. The investigations now need to be extended in two directions: 1. The binding specificity should be examined in more in vivo-like systems, for instance isolated hepatocytes. Then a possible correlation between toxic effects and binding patterns could be investigated. 2. Target proteins should be identified and possible effects of the binding on their biological function tested.

d. Toxic effects of benzene and some of its metabolites on bone marrow in mice

The bone marrow is a complex organ where differentiation and proliferation from multipotent stem cells to several kinds of blood cells take place continuously. As reviewed above, it appears that all cell lines in the bone marrow are affected by benzene exposure. We tried to design our investigations on the bone marrow in such a way that they would cover some very different functions of this organ (V, VI). Bone marrow from one tibia from each mouse was taken, and the parameters investigated were: 1. Overall cellularity (number of nucleated cells per tibia). 2. Absolute and relative amount of colony forming granulopoietic stem cells (CFU-C) (measured as the number of colonies forming in agar culture after stimulation with "colony stimulating factors" (CSF)). 3. Genotoxicity on the erythropoietic cell line (measured as the occurrence of micronuclei in polychromatic erythrocytes (PCE)). Thus, it would be possible to find various effects of benzene and of

some of its metabolites on the bone marrow, and also to get some insight into the sequence of the occurrence of different adverse effects.

When benzene was injected subcutaneously at a daily dose of 440 mg/kg body weight for 1-6 days, all parameters studied were severely affected (V, VI). The overall cellularity was suppressed by as much as 95% after 4-6 injections. The number of CFU-C/tibia was even more suppressed, leading to a decrease also in the number of CFU-C/ 10^5 cells. Micronuclei occurred in the PCE already after 1 injection and a maximum was reached after 3 injections. Then as many as 10% of the PCE contained micronuclei (VI). After more than 3 injections, a decrease was observed in the frequency of micronuclei, probably reflecting cell death before the PCE-stage during erythropoiesis.

In other experiments benzene was injected subcutaneously at 6 daily doses ranging between 0.7 and 440 mg/kg body weight (V, VI). The cellularity decreased at 3.5 mg/kg and higher doses, but the effect was dramatic only at the highest dose. The number of CFU-C/tibia was, again, more affected than was the cellularity, and a significant reduction of CFU-C/ 10^5 nucleated cells was observed already at the lowest dose. Micronuclei occurred in the PCE only at the two highest doses (88 and 440 mg/kg body weight).

In summary, the dose-response studies on the effects of benzene revealed that all parameters investigated were affected. Most sensitive was the number of CFU-C/ 10^5 nucleated cells and CFU-C/tibia. The overall cellularity also decreased at low benzene doses, although the effect was not as pronounced as the effect on CFU-C. Micronuclei in PCE could be demonstrated only at relatively high benzene doses.

The three benzene metabolites phenol, benzene dihydrodiol and hydroquinone were also tested for their hemotoxic effects (V, VI). Phenol (at doses up to 6 x 100 mg/kg body weight) and benzene dihydrodiol (at doses up to 6 x 300 mg/kg body weight) had no, or very

mild, toxic effects on any of the parameters studied. Hydroquinone, on the other hand, gave dose-dependent effects in the different tests (VI). The overall cellularity of the tibia was elevated at low doses, was about the same as in untreated mice at 6 x 50 mg/kg, and was reduced by about 60% at the highest dose (6 x 100 mg/kg). The number of CFU-C/tibia responded in a similar way as the cellularity, but the number of CFU-C/ 10^5 nucleated cells was higher after hydroquinone injections than in untreated animals (with the exception of the lowest dose). The number of micronuclei was elevated at doses above 6 x 20 mg/kg. It increased rather slowly up to 6 x 50 mg/kg, and then a dramatical rise occurred with a maximum at a dose of 6 x 70 - 6 x 80 mg/kg body weight. The reason for the sudden increase in the number of micronuclei in this dose interval might be that some detoxifying enzyme system (UDP-glucuronosyl transferase or glutathione transferases) became saturated, or that some cofactor (UDP-glucuronic acid or glutathione) was depleted, leading to appreciable amounts of free hydroquinone. A sharp decrease in cellularity and CFU-C/tibia occurred in the same dose range (6 x 50 - 6 x 80 mg/kg body weight).

A comparison of the effect of benzene and hydroquinone on the bone marrow shows that all parameters studied were affected by both compounds, but that several differences in the response were noted. The cellularity and the number of CFU-C/tibia were suppressed by benzene and by the highest doses of hydroquinone, while low doses of hydroquinone rather increased these levels. Benzene decreased the CFU-C/tibia relatively more than it decreased the cellularity, while the opposite was true for hydroquinone. Benzene induced the formation of micronuclei only at doses where a suppression of cellularity and CFU-C was pronounced, while hydroquinone gave micronuclei at doses rather elevating these parameters.

As indicated in the review, there are some indications that benzene metabolites accumulate in the bone

marrow following exposure to benzene (33, 35). The information is, however, not sufficient to predict the concentration of hydroquinone in the bone marrow resulting from a dose of benzene. Neither do we have enough information to predict the concentration of hydroquinone in bone marrow following injections of hydroquinone in vivo. It is, therefore, difficult to estimate to what extent hydroquinone may contribute to the toxic effects of benzene. The results presented here clearly show that hydroquinone is a hemotoxic metabolite of benzene, and it seems likely that at least a part of the benzene-induced toxicity is caused by metabolic formation of hydroquinone. However, the differences in the responses suggest that some other metabolite(s), or benzene itself, also possesses hemotoxic properties. The observations presented in V and VI support a toxicological relevance of the major reactive metabolites of benzene in vitro, i.e. p-benzosemiquinone and p-benzoquinone (II, III).

Summary

Benzene monooxygenase activity in rat liver microsomes was enhanced about 3-fold after pretreatment of the animals with benzene or phenobarbital. The control and benzene-induced activities increased following addition of metyrapone or Renex 690 to the incubation mixtures, while the phenobarbital-induced activity was almost completely inhibited. It was concluded that benzene-pretreatment induces the constitutive form(s) of benzene monooxygenase, or a form (forms) with very similar properties, while phenobarbital-pretreatment induces monooxygenases, accepting benzene as substrate, with properties different from those of the constitutive forms.

The metabolic activation of benzene in rat liver microsomes is a multi-step process, including benzene oxide, phenol, and hydroquinone as intermediates, and p-benzosemiquinone and/or p-benzoquinone as quantita-

tively important protein-binding metabolites. The binding specificity of benzene and phenol metabolites to microsomal proteins was investigated, and most binding was associated with a component with a molecular weight of about 72,000. The binding patterns of benzo(a)pyrene and 3-methylcholanthrene metabolites (with different types of epoxides as likely binding species) were very different from those of benzene and phenol. Differences in metabolic activation pathways therefore appear to be reflected by differences in binding patterns.

Subcutaneous injections of benzene into mice produced severe toxic effects on the bone marrow. The cellularity and the absolute and relative amount of colony forming granulopoietic stem cells decreased, and micronucleus formation in erythropoietic cells was induced. Phenol and benzene dihydrodiol produced no, or very mild, toxic effects, but the bone marrow parameters measured were affected by hydroquinone. At least a part of the benzene-induced toxicity on the bone marrow seems to be caused by hydroquinone formation, but other metabolites of benzene, or benzene itself, probably also contribute to toxicity.

REFERENCES

1. Santesson, C.G. Arch. Hyg. Berl., 31:336, 1897.
2. Selling, L. Bull. Johns Hopkins Hosp., 21:33, 1910.
3. Selling, L. Johns Hopkins Hosp. Rep., 17:83, 1916.
4. Hamilton, A. JAMA, 78:627, 1922.
5. Hamilton, A. J. Ind. Hyg., 10:227, 1928.
6. Berlin, M., Gage, J.C. and Johnson, E. Work-environm.-hlth., 11:1, 1974.
7. Sherwood, R.J. Ann. Occup. Hyg., 15:409, 1972.
8. Stichting CONCAWE, The Hague. Report 1/73, February, 1973.
9. Berlin, M., Fredga, K., Gage, J.C., Lagesson, V., Reitalu, J. and Tunek, A. Report nr. 750616 from the departments of Environmental Health and Genetics, University of Lund, 1975 (summary in English).
10. Laskin, S. and Goldstein, B.D. J. Tox. Env. Health, suppl. 2, 1977.
11. U.S. Department of Health, Education and Welfare, Washington, D.C. NIOSH 74, 1974.
12. Berlin, M., Holm, S. and Korsell, M. Report nr. 780717, from the department of Environmental Health, University of Lund, 1978.
13. Aksoy, M. New Istanbul Contrib. Clin., 12:3, 1977.
14. Vigliani, E.C. Ann. N.Y. Acad. Sci., 271:143, 1977.
15. Aksoy, M., Dincol, K., Erdem, S. and Dincol, G. Am. J. Med., 52:160, 1972.
16. Gage, J.C., Lagesson, V. and Tunek, A. Ann. Occup. Hyg., 20:127, 1977.
17. Berlin, M., Fredga, K., Gullberg, B., Holm, S., Knutsson, P., Reitalu, J. and Tunek, A. Report nr. 771018 from the departments of Environmental Health and Genetics, University of Lund, 1977 (summary in English).
18. Fredga, K., Reitalu, J. and Berlin, M. In: Genetic Damage in Man Caused by Environmental Agents (Berg, E.K., ed.) p. 187, Academic Press, New York, 1979.
19. Picciano, D. Env. Res., 19:33, 1979.
20. Hunter, C.G. and Blair, D. Ann. Occup. Hyg., 15:193, 1972.

21. Hunter, C.G. *Ann. Occup. Hyg.*, 9:191, 1966.
22. Römmelt, H. and Dirnagl, K. *Münch. Med. Wochenschr.*, 119:367, 1977.
23. Teisinger, J., Bergerova-Fiserova, V. and Kudrna, J. *Procovni lekarstvi*, 4:175, 1952 (Polish; summary in English).
24. Gut, I. *Arch. Toxicol.*, 35:195, 1976.
25. Hanke, J., Dutkiewicz, T. and Piotrowski, I. *Med. Pracy*, 12:413, 1961.
26. Dutkiewicz, T. and Tyras, H. *Br. J. Indust. Med.*, 24:330, 1967.
27. Dutkiewicz, T. and Tyras, H. *Arch. Gewerbepathol. Gewerbehyg.*, 24:253, 1968.
28. Duvoir, M.R., Fabre, A. and Derobert, L. *Arch. Mal. Prof.*, 7:77, 1946.
29. Srbova, J., Teisinger, J. and Skramovsky, S. *Arch. Ind. Hyg. Occup. Med.*, 2:1, 1950.
30. Nordisk ekspertgruppe for graensevaerdokumentation. Copenhagen, 1979 (Danish).
31. Sato, A., Fujiwara, Y. and Nakajima, T. *Sangyo Igaku*, 16:30, 1974.
32. Berlin, M., Gage, J.C., Gullberg, B., Holm, S., Knutsson, P. and Tuneek, A. *Scand. J. Work Env. Health*, 6:104, 1980.
33. Andrews, L.S., Lee, E.W., Witmer, C.M., Kocsis, J.J. and Snyder, R. *Biochem. Pharmacol.*, 26:293, 1977.
34. Bergman, K. *Scand. J. Work Env. Health*, 5:suppl. 1, 1979.
35. Rickert, D.E., Baker, T.S., Bus, J.S., Barrow, C.S. and Irons, R.D. *Toxicol. Appl. Pharmacol.*, 49:417, 1979.
36. Lutz, W.K. and Schlatter, C. *Chem.-Biol. Interact.*, 18:241, 1977.
37. Snyder, R., Lee, E.W. and Kocsis, J.J. *Res. Commun. Chem. Pathol. Pharm.*, 20:191, 1978.
38. Irons, R.D., Dent, J.G., Baker, T.S. and Rickert, D.E. *Chem.-Biol. Interact.*, 30:241, 1980.
39. Abou-el-Marakem, M.M., Millburn, P., Smith, R.L. and Williams, R.T. *Biochem. J.*, 105:1269, 1967.

40. Rusch, G.M., Leong, B.K.J. and Laskin, S. J. Tox. Env. Health, suppl. 2:23, 1977.
41. Schultzen, O. and Naunyn, B. Arch. Anat. Physiol. Lpz., 349, 1867.
42. Porteous, J.W. and Williams, R.T. Biochem. J., 44:46, 1949.
43. Porteous, J.W. and Williams, R.T. Biochem. J., 44:56, 1949.
44. Parke, D.V. and Williams, R.T. Biochem. J., 54:231, 1953.
45. Parke, D.V. and Williams, R.T. Biochem. J., 55:337, 1953.
46. Capel, I.D., French, M.R., Millburn, P., Smith, R.L. and Williams, R.T. Xenobiotica, 2:25, 1972.
47. Kao, J. and Bridges, J.W. Xenobiotica, 9:141, 1979.
48. Ayengar, P., Hayaishi, O., Nakajima, M. and Tomida, I. Biochem. Biophys. Acta, 33:11, 1959.
49. Jerina, D.M., Ziffer, H. and Daly, J.W. J. Am. Chem. Soc., 92:1056, 1970.
50. Sato, T., Fukuyama, T., Suzuki, T. and Yoshikawa, H. J. Biochem., 53(1):23, 1963.
51. Garton, G.A. and Williams, R.T. Biochem. J., 44:234, 1949.
52. Garton, G.A. and Williams, R.T. Biochem. J., 43:206, 1948.
53. Snyder, R. In: Symposium on Toxicology of Benzene and Alkylbenzenes (Braun, D., ed.) p. 44, Industrial Health Foundation, Pittsburgh, 1974.
54. Oehme, F. Thesis, University of Missouri, Columbia. University Microfilms, Inc., Ann Arbor, Mich., 1969.
55. Cornish, H.H. and Ryan, R.R. Toxicol. Appl. Pharmacol., 7:767, 1965.
56. Sakamoto, A., Inamori, K., Watanabe, I. and Fukiware, E. Osaka Daigaku Zasshi, 9:345, 1957.
57. Posner, H.S., Mitoma, C. and Udenfriend, S. Arch. Biochem. Biophys., 94:269, 1961.
58. Snyder, R., Uzuki, F., Gonasun, L., Bromfeld, E. and Wells, A. Toxicol. Appl. Pharmacol., 11:346, 1967.

59. Harper, C., Drew, R.T. and Fouts, J.R. Drug Metab. Dispos., 3:381, 1975.
60. Sato, R. and Omura, T. (eds.), Cytochrome P-450, Academic Press, New York, 1978.
61. Mannering, G.J. In: Fundamentals of Drug Metabolism and Disposition (La Du, B.N., Mandel, H.G. and Way, E.L., eds.) chap. 12, Williams and Wilkins, Baltimore, 1971.
62. Thorgeirsson, S.S. and Nebert, D.W. In: Advances in Cancer Research (Klein, G. and Weinhouse, S., eds.) vol. 25, p. 149, Academic Press, New York, 1977.
63. Coon, M.J. and Vatsis, K.P. In: Polycyclic Hydrocarbons and Cancer (Gelboin, H.V. and Ts'o, P.O.P., eds.) p. 335, Academic Press, New York, 1978.
64. Gonasun, L.M., Witmer, C.M., Kocsis, J.J and Snyder, R. Toxicol. Appl. Pharmacol., 26:398, 1973.
65. Jerina, D., Daly, J., Witkop, B., Zaltzman-Nirenberg, P. and Udenfriend, S. Arch. Biochem. Biophys., 128:176, 1968.
66. Oesch, F., Bentley, P., Platt, K.L. and Golan, M. Arch. Biochem. Biophys., 199:538, 1980.
67. Sammett, D., Lee, E.W., Kocsis, J.J. and Snyder, R. J. Tox. Env. Health, 5:785, 1979.
68. Drew, R.T. and Fouts, J.R. Toxicol. Appl. Pharmacol., 27:183, 1974.
69. Andrews, L.S., Sasame, H.A. and Gillette, J.R. Life Sci., 25:567, 1979.
70. Sloane, N.H. Biochim. Biophys. Acta, 107:599, 1965.
71. Conney, A.H. Pharmacol. Rev., 9:317, 1967.
72. Fujita, T., Shoeman, D.W. and Mannering, G.J. J. Biol. Chem., 248:2192, 1973.
73. Ryan, D., Thomas, P.E., Korzeniowski, D. and Levin, W. J. Biol. Chem., 254:1365, 1979.
74. Alvares, A.P. In: Microsomes and Drug Oxidations (Ullrich, V., Roots, I., Hildebrandt, A., Estabrook, R.W. and Conney, A.H., eds.) p. 476, Pergamon Press, Oxford, 1977.
75. Parke, D.V. and Rahman, H. Biochem. J., 123:9, 1971.

76. Ullrich, V., Weber, P. and Wollenberg, P. *Biochem. Biophys. Res. Commun.*, 64:808, 1975.
77. Ullrich, V., Frommer, U. and Weber, P. *Hoppe-Seyler's Z. Physiol. Chem.*, 354:514, 1973.
78. Wisniewska-knypl, J.M., Jablonska, J.K. and Piotrowski, J.K. *Br. J. Ind. Med.*, 32:42, 1975.
79. Saito, F.U., Kocsis, J.J. and Snyder, R. *Toxicol. Appl. Pharmacol.*, 26:209, 1973.
80. Norpoth, K., Witting, U. and Springorum, M. *Int. Arch. Arbeitsmed.*, 33:315, 1974.
81. Drew, R.T., Fouts, J.R. and Harper, C. In: *Symposium on Toxicology of Benzene and Alkylbenzenes* (Braun, D., ed.) p. 17, Industrial Health Foundation, Pittsburgh, 1974.
82. Timbrell, J.A. and Mitchell, J.R. *Xenobiotica*, 7:415, 1977.
83. Fouts, J.R. and Rogers, L.A. *J. Pharmacol. Exp. Ther.*, 147:112, 1965.
84. Orrenius, S. and Ericsson, J.L. *J. Cell. Biol.*, 28:181, 1966.
85. Gonasun, L. Ph.D. Thesis, Jefferson Medical College, Philadelphia, Pennsylvania, 1971.
86. Alvares, A.P., Schilling, G., Levin, W. and Kuntzman, R. *Biochem. Biophys. Res. Commun.*, 29:521, 1967.
87. Gelboin, H. and Solokoff, L. *Science*, 134:611, 1961.
88. Kato, R., Jondorf, W.R., Loeb, L.A., Ben, T. and Gelboin, H. *Mol. Pharmacol.*, 2:171, 1966.
89. Bresnick, E., Brand, R. and Knight, J.A. *Biochim. Biophys. Acta*, 114:227, 1966.
90. Gelboin, H.V., Wortham, J.J. and Wilson, R.G. *Nature*, 214:281, 1967.
91. Gelboin, H.V. In: *Handbook of Experimental Pharmacology*, (Brodie, B.B. and Gillette, J.R., eds.) part 2, p. 431, Springer-Verlag, Berlin, 1971.
92. Capdevila, J., Jakobsson, S., Jernström, S., Helia, O. and Orrenius, S. *Cancer Res.*, 35:2820, 1975.
93. Ikeda, M. and Ohtsuki, H. *Toxicol. Appl. Pharmacol.*, 20:30, 1971.

94. Gill, D.P., Kempen, R.R., Nash, J.B. and Ellis, S.
Life Sci., 25:1633, 1979.
95. Kocsis, J.J., Harkaway, S., Santoyo, M.C. and Snyder,
R. Science, 160:427, 1968.
96. Stock, B.H., Hansen, A.R. and Fouts, J.R. Toxicol.
Appl. Pharmacol., 16:728, 1970.
97. Heim, W., Appelman, G.D. and Pyfrom, J.T. Science,
122:693, 1955.
98. Nomiyama, K. Med. J. Shinshu Univ., 7:41, 1962.
99. Nomiyama, K. Bull. Tokyo Med. Dent. Univ., 11:297,
1964.
100. Abramova, Zh.I. and Gadaskina, I.D. Fed. Proc. Proc.
Trans. Suppl., 25:T91, 1966.
101. Ikeda, M., Ohtsuji, H. and Imamura, T. Xenobiotica, 2:
101, 1972.
102. Sato, A. and Nakajima, T. Toxicol. Appl. Pharmacol.,
48:249, 1979.
103. Jonek, J., Olkowski, Z. and Zieleznik, B. Acta histo-
chem., 20:286, 1965.
104. Iannaccone, A. and Ciccella, G. Sci. med. ital. Ser. 2,
8:59, 1959-60.
105. Jonek, J., Kaminski, M., Latkowska, H. and Kaminska, O.
Acta histochem., 44:15, 1972.
106. Sroczynski, M., Kaminski, J. and Jonek, J. Arch. mal.
prof., 30:403, 1969.
107. Jonek, J., Kaminski, M., Konecki, J. and Gruszecka, B.
Exp. Pathol., 11:51, 1975.
108. Jonek, J., Grzybek, H., Kaminski, M. and Kochanska, D.
Int. Arch. Gewerbepath. Gewerbehyg., 22:342, 1966.
109. Browning, E. Toxicity and Metabolism of Industrial
Solvents. 739 p., Elsevier, Amsterdam, 1965.
110. Mallory, T.G., Gall, E.A. and Brickley, W.J. J. ind.
hyg. toxicol., 21:355, 1939.
111. Jonek, J., Kaminski, M., Grzybek, H. and Panz, B. Acta
med. pol., 12:241, 1971.
112. Wirtschafter, Z.T. and Cronyn, M.W. Arch. environ.
health, 9:180, 1964.
113. Carpenter, C.P., Shaffer, C.B., Weil, C.S. and Smyth,
H.F., Jr. J. Ind. Hyg. Toxicol., 28:69, 1944.

114. Kimura, E.T., Ebert, D.M. and Dodge, P.W. Toxicol. Appl. Pharmacol., 19:699, 1971.
115. Snyder, R. and Kocsis, J.J. CRC Critical Review of Toxicology, 3:265, 1975.
116. Snyder, R., Lee, E.W., Kocsis, J.J. and Witmer, C.M. Life Sci., 21:1709, 1977.
117. Gerarde, H.W. Arch. Ind. Health, 13:468, 1956.
118. Weiskotten, H.G., Schwartz, S.C. and Steensland, H.S. J. Med. Res., 35:63, 1916.
119. Snyder, C.A., Goldstein, B.D., Sellakumar, A.R., Bromberg, I., Laskin, S. and Albert, R.E. Toxicol. Appl. Pharmacol., 54:323, 1980.
120. Snyder, C.A., Goldstein, B.D., Sellakumar, A., Albert, R.E. and Laskin, S. Toxicol. Appl. Pharmacol., 45, abstract 106, 1978.
121. Deichmann, W.B., MacDonald, W.E. and Bernal, E. Toxicol. Appl. Pharmacol., 5:201, 1963.
122. Aksoy, M., Dincol, K., Akgun, T., Erdem, S. and Dincol, G. Br. J. Ind. Med., 28:296, 1971.
123. Savilahti, M. Arch. Gewerbepathol. Gewerbehyg., 15:147, 1956.
124. Hernberg, S., Savilahti, M., Ahlman, K. and Asp, S. Br. J. Ind. Med., 23:204, 1966.
125. Goldwater, L.J. J. Lab. Clin. Med., 26:957, 1941.
126. Wilson, R.H. J. Lab. Clin. Med., 27:1517, 1942.
127. Kissling, M. and Speck, B. Helv. Med. Acta, 36:59, 1969.
128. Boje, H., Benkel, W. and Heiniger, H.J. Blut, 21:250, 1970.
129. Lee, E.W., Kocsis, J.J. and Snyder, R. Toxicol. Appl. Pharmacol., 27:431, 1974.
130. Lee, E.W., Kocsis, J. and Snyder, R. Res. Commun. Chem. Pathol. Pharmacol., 5(2):547, 1973.
131. Irons, R.D., Heck, H. d'A., Moore, B.J. and Muirhead, K.A. Toxicol. Appl. Pharmacol., 51:399, 1979.
132. Steinberg, B. Blood, 4:550, 1949.
133. Uyeki, E.M., El Ashkar, A., Shoeman, D.W. and Bisel, T.U. Toxicol. Appl. Pharmacol., 40:49, 1977.
134. Lignac, G.O.E. Klin. Wochenschr., 12:109. 1932.

135. Girard, R. and Revol, L. *Nouv. rev. fr. Haematol.*, 10: 477, 1970.
136. Vigliani, E.C. and Saita, G. N. *Engl. J. Med.*, 271:872, 1964.
137. Infante, P.F., Rinsky, R.A., Wagoner, J.K. and Young, R.J. *Lancet*, 2:76, 1977.
138. Aksoy, M., Erdem, S. and Dincol, G. *Acta Haematol.*, 55:65, 1976.
139. Tareef, E.M., Kontchalovskaya, N.M. and Zorina, L.A. *Acta Unio Int. Contra Cancrum*, 19:751, 1963.
140. Goguel, A., Cavigneaux, A. and Bernard, J. *Nouv. rev. fr. Haematol.*, 7:465, 1967.
141. McMichael, A.J., Spirtas, R., Kupper, L.L. and Gamble, J.F. *J. Occup. Med.*, 17:234, 1975.
142. Goldstein, B.D. *J. Tox. Env. Health*, suppl. 2:69, 1977.
143. Vigliani, E.C. and Forni, A. *Environ. Res.*, 11:122, 1976.
144. Aksoy, M., Dincol, K., Erdem, S., Akgun, T. and Dincol, G. *Br. J. Ind. Med.*, 29:56, 1972.
145. Kinoshita, Y., Terada, H. and Saito, H. *J. Jpn. Haematol. Soc.*, 85, 1965.
146. Sellyei, M. and Kelemen, E. *Eur. J. Cancer*, 7:83, 1971.
147. Ott, M.G., Townsend, F.C., Fishbeck, W.A. and Langner, R.A. *Arch. Env. Health*, 33:3, 1978.
148. Tabershaw, I.R. and Lamm, S.H. *Lancet*, 2:867, 1977.
149. McMichael, A.J., Spirtas, R., Gamble, J.F. and Tousey, P.M. *J. Occup. Med.*, 18:178, 1976.
150. Monson, R.R. and Nakano, K.K. *Am. J. Epidemiol.*, 103: 297, 1976.
151. Townsend, J.C., Ott, M.G. and Fishbeck, W.A. *J. Occup. Med.*, 20:543, 1978.
152. Thorpe, J.J. *J. Occup. Med.*, 16:375, 1974.
153. Dobrokhotoy, V.B. and Enikeev, M.I. *Gig. Sanit.* 1, 32, 1977.
154. Siou, M.G. and Conan, L. *Cah. Notes Doc.*, 69:433, 1977.
155. Hite, M., Pecharo, M., Smith, I. and Thornton, S. *Mut. Res.*, 77:149, 1980.
156. Lyon, J.P. Ph.D. Thesis, University of California, 1975.
157. Dobashi, Y. *Jpn. J. Ind. Health*, 16:453, 1974.

158. Koizumi, A., Dobashi, Y., Tachibana, Y., Tsuda, K. and Katsunuma, H. *Ind. Health*, 12:23, 1974.
159. Speck, B., Schnider, Th., Gerber, U. and Noeschlin, S. *Schweiz. Med. Wochenschr.*, 96:1274, 1966.
160. Kissling, M. and Speck, B. *Helv. Med. Acta*, 36:59, 1972.
161. Morimoto, K. *Jap. J. Ind. Health*, 17:106, 1975.
162. Morimoto, K. and Wolff, S. *Cancer Res.*, 40:1189, 1980.
163. Diaz, M., Fijtman, N., Carricarte, V., Braier, L. and Diez, J. *In Vitro*, 15:172 (abstract 23), 1979.
164. Tice, R.R., Costa, D.L. and Drew, R.T. *Proc. Natl. Acad. Sci. USA*, 77:2148, 1980.
165. Dean, B.J. *Mutat. Res.*, 47:75, 1978.
166. Mitelman, F., Brandt, L. and Nilsson, P.G. *Blood*, 52:1229, 1978.
167. Forni, A. and Moreo, L. *Eur. J. Cancer*, 5:459, 1969.
168. Forni, A. and Moreo, L. *Eur. J. Cancer*, 3:251, 1967.
169. Forni, A., Cappellini, A., Pacifico, E. and Vigliani, E.C. *Arch. Environ. Health*, 23:385, 1971.
170. Girard, R., Mallein, M.L., Bertholon, J., Coeur, P. and Evreux, J. *Arch. Mal. Prof.*, 31:31, 1970.
171. Fredga, K., Dävring, L., Sunner, M., Bengtsson, B.O., Elinder, C.G., Sigtryggsson, P. and Berlin, M. Report nr. 790320 from the departments of Environmental Health and Genetics, University of Lund, 1979 (summary in English).
172. Gofmekler, V.A. *Hyg. Sanit.*, 33:327, 1968.
173. Murray, F.J., John, J.A., Rampy, L.W., Kuna, R.A. and Schwetz, B.A. *Am. Ind. Hyg. Ass. J.*, 40:993, 1979.
174. Green, J.D., Leong, B.K.J. and Laskin, S. *Toxicol. Appl. Pharmacol.*, 48:9, 1978.
175. Watanabe, G.-I. and Yoshida, S. *Acta Med. Biol.*, 17(4):285, 1970.
176. Nawrot, P.S. and Staples, R.E. *Teratology*, 19:41A (abstract), 1979.
177. White, W.C. and Gammon, A.M. *Trans. Assoc. Am. Physicians*, 29:332, 1914.
178. Winternitz, M.C. and Hirschfelder, A.D. *J. Exp. Med.*, 11:657, 1913.
179. Simonds, J.J. and Jones, H.M. *J. Med. Res.*, 33:197, 1915.

180. Irons, R.D. and Moore, B.J. Res. Comm. Chem. Path. Pharm., 27:147, 1980.
181. Lange, A., Smolik, R., Zatonski, W. and Glazman, H. Int. Arch. Arbeitsmed., 31:45, 1973.
182. Lange, A., Smolik, R., Zatonski, W. and Szymanska, J. Int. Arch. Arbeitsmed., 31:37, 1973.
183. Smolik, K., Grzylek-Hrynecqicx, K., Lange, A. and Zatonski, W. Int. Arch. Arbeitsmed., 31:243, 1973.
184. Jollow, D.J., Kocsis, J.J., Snyder, R. and Vainio, H. (eds.). Biological Reactive Intermediates. Formation, Toxicity and Inactivation. Plenum Press, New York, 1977.
185. Gillette, J.R., Mitchell, J.R. and Brodie, B.B. Annu. Rev. Pharmacol., 14:271, 1974.
186. Heidelberger, C. Annu. Rev. Biochem., 44:79, 1975.
187. Jerina, D.M. and Daly, J.W. Science, 185:573, 1974.
188. Gelboin, H.V. and Ts'o, P.O.P. (eds.). Polycyclic Hydrocarbons and Cancer. 1. Environment, Chemistry and Metabolism. Academic Press, New York, 1978.
189. Miller, E.C. Cancer Res., 38:1479, 1978.
190. Weisburger, E.K. Annu. Rev. Pharmacol. Toxicol., 18: 395, 1978.
191. Mitchell, J.R. Fed. Proc., 30:abstract 2044, 1971.
192. Mitchell, J.R. and Jollow, D.J. Gastroenterology, 68: 392, 1975.
193. Nomiyama, K. Ind. Health, 3:53, 1965.
194. Harrison, K. and Randoll, F.W. Quart. J. Exp. Physiol., 74:141, 1948.
195. Dexter, T.M., Allen, T.D. and Lajtha, L.G. J. Cell Physiol., 91:335, 1977.
196. Pulkrabek, P., Kinoshita, T. and Jeffery, A.M. Am. Ass. Cancer Res., 21:107 (abstract 427), 1980.
197. Morimoto, K. Jap. J. Ind. Health, 17:166, 1975.
198. Morimoto, K., Koizumi, A., Tachibana, Y. and Dobashi, Y. J. Radiat. Res., 18:51 (abstract 3-A-10), 1977.
199. Tryfiates, G.P. Biochem. Pharmacol., 20:1669, 1971.
200. Tryfiates, G.P. and Choulis, N.H. J. Pharm. Sciences, 61:465, 1972.

201. Forte, F.J., Cohen, H.S., Rosman, J. and Freedman, M. L. Blood, 47:145, 1976.
202. Freedman, M.L., Wildman, J.M., Rosman, J., Eisen, J. and Greenblatt, D.R. Br. J. Haematol., 34:49, 1977.
203. Freedman, M.L., Cohen, H.S., Ibrahim, N.G. and Gruenspecht, N. Env. Res., 18:291, 1979.
204. Kahn, H. and Muzyka, V. Work-environm.-hlth., 10:140, 1973.
205. Burchell, P., Bentley, P. and Oesch, F. Biochim. biophys. Acta, 444:531, 1976.
206. Leibman, K.C., Mol. Pharmacol., 5:1, 1969.
207. Kahl, G.F., Minck, K. and Netter, K.J. Drug Metab. Dispos., 1:191, 1973.
208. Dutton, G.J. In: Handbook of Experimental Pharmacology, Concepts in Biochemical Pharmacology, Part 2 (Brodie, B.B. and Gillette, J.R., eds.) p. 378, Springer-Verlag, Berlin, 1971.
209. Dybing, E., Nelson, S.D., Mitchell, J.R., Sasame, H.A. and Gillette, J.R. Mol. Pharmacol., 12:911, 1976.
210. Marks, F. and Hecker, E. Biochim. biophys. Acta, 187: 250, 1969.
211. Bolt, H.M. and Kappus, H. J. Steroid. Biochem., 5:179, 1974.
212. Winterbourn, C.C., French, J.K. and Claridge, R.F.C. FEBS Lett., 94:269, 1978.
213. McCord, J.M. and Fridovich, I. J. Biol. Chem., 244: 6049, 1969.
214. Jerina, D.M., Yagi, H., Thakker, D.R., Lehr, R.E., Wood, A.W., Levin, W. and Conney, A.H. In: Microsomes, Drug Oxidations, and Chemical Carcinogenesis, Volume II (Coon, M.J., Conney, A.H., Estabrook, R.E., Gelboin, H.V., Gillette, J.R. and O'Brien, P.J., eds.) p. 1041, Academic Press, New York, 1980.
215. Stoming, T.A., Bornstein, W. and Bresnick, E. Biochem. Biophys. Res. Commun., 79:461, 1977.
216. Wood, A.W., Chang, R.L., Levin, W., Thomas, P.E., Ryan, D., Stoming, T.A., Thakker, D.R., Jerina, D.M. and Conney, A.H. Cancer Res., 38:3398, 1978.

UNIQUE BEHAVIOUR OF BENZENE MONO-OXYGENASE: ACTIVATION BY DETERGENT AND DIFFERENT PROPERTIES OF BENZENE- AND PHENOBARBITAL- INDUCED MONO-OXYGENASE ACTIVITIES

ANDERS TUNEK* and FRANZ OESCH

Section on Biochemical Pharmacology, The University of Mainz, Obere Zahlbacher Straße 67,
D-6500 Mainz, West Germany

(Received 10 January 1979; accepted 1 June 1979)

Abstract—The benzene mono-oxygenase present in liver microsomes from untreated rats was activated 3.5-fold by the detergent Renex 690. This is the first report of an activation of a microsomal mono-oxygenase by detergent. Metyrapone also activated benzene mono-oxygenase, albeit not as strongly (1.5-fold). Treatment of the rats with benzene or phenobarbital induced the benzene mono-oxygenase activity about 3-fold. The benzene-induced form was also activated by the detergent and by metyrapone. However, the phenobarbital-induced activity was inhibited by both modulators.

When the mono-oxygenase activity was measured with 7-ethoxycoumarin as a model substrate, which is accepted by many microsomal mono-oxygenases, the phenobarbital-induced activity was inhibited strongly (~80 per cent) by metyrapone or Renex 690; however, both the benzene-induced and the control activity were inhibited only weakly (~30 per cent).

The results provide evidence for substantially different properties of the phenobarbital-induced mono-oxygenase(s) compared to control mono-oxygenase(s). Moreover, benzene appears to be a type of inducer different from the known types and of special interest since, whether assayed with benzene or 7-ethoxycoumarin as substrates, and Renex 690 or metyrapone as modulators, the benzene-induced mono-oxygenase activity possesses characteristics resembling the controls rather than that induced by phenobarbital.

Benzene is a component of most motor fuels and therefore a ubiquitous environmental pollutant [1]. The toxicity of benzene is specifically directed towards the bone marrow [2, 3]. Some evidence indicates that metabolism of benzene is required to produce the toxic effects [4]. It is thought that such metabolism-induced toxic effects originate from the formation of reactive intermediates, which are able to interact covalently with macromolecules, thereby disturbing the cellular functions. The formation of such reactive intermediates, especially epoxides, has been extensively studied for some aromatic polycyclic hydrocarbons [5-8].

Recently, we investigated the irreversible binding *in vitro* of benzene metabolites to microsomal proteins. We found that, although benzene oxide clearly is formed, it was not this epoxide but rather a secondary metabolite, namely a metabolite of phenol, which was mainly responsible for this binding [9].

In the present study we show some unusual properties of the rat liver benzene mono-oxygenase, the enzyme responsible for the first step in the metabolic activation of benzene. This enzyme has been shown to be a cytochrome P-450-dependent mono-oxygen-

ase [10], and hydroxylation proceeds, at least to a large extent, via benzene oxide [9]. Furthermore, some properties of the benzene-induced mono-oxygenase activity are investigated and compared to those of PB† induced mono-oxygenase.

EXPERIMENTAL SECTION

7-Ethoxycoumarin was synthesized as described [11]. Metyrapone was a gift from Ciba-Geigy, Basel, Switzerland. All chemicals, obtained from commercial sources, were of analytical grade. [¹⁴C] Benzene and [¹⁴C] phenol from the Radiochemical Centre, Amersham, U.K., had specific activities of 107 and 35 mCi/mmol, respectively, and were > 99 per cent radiochemically pure. The detergent Renex 690 was purchased from Atlas-Chemie, Essen, West Germany, and 7-hydroxycoumarin from EGA-Chemie, Steinheim, West Germany.

Adult male Sprague-Dawley rats (180-280 g) were obtained from Versuchstier-Zuchtanstalt Wiga, Sulzfeld, West Germany, and kept in plastic cages with hardwood bedding under standardized conditions of light (light-dark cycle: 7 a.m. to 7 p.m.) and temperature (21-24°) for at least 4 days before treatment. They had free access to Aldromin pellets (Samen-Schmitt-Jakobi, Frankfurt/M., West Germany) and tap water. Care was taken to avoid any environmental effects such as cigarette smoke, insecticides, noise, etc. Animals were always treated

* Permanent address: Institute of Environmental Health, University of Lund, Sölvegatan, 21, S-223 62 Lund, Sweden.

† Abbreviations used: PB, phenobarbital.

between 8 and 10 a.m. The PB-induced animals received 80 mg PB per kg body weight, dissolved in 0.9% NaCl, intraperitoneally for three consecutive days and were sacrificed on the fourth day. The benzene-induced animals received 1.1 g benzene per kg body weight subcutaneously 24 and 18 hr before being sacrificed, as described previously [10]. Control animals received either intraperitoneal saline injections at the same time as the PB-treated, or no injection at all.

In this study mono-oxygenase activity with benzene and 7-ethoxycoumarin as substrates, respectively, is expressed as phenol and 7-hydroxycoumarin formed (often referred to as "benzene hydroxylase" and "ethoxycoumarin de-ethylase"). The preparation of rat liver microsomes, incubations and measurement of phenol formation were performed as has been described [9], except that the incubation time was reduced to 30 min and metyrapone, when added, was dissolved in 10 μ l acetone (total incubation volume 3 ml). To all controls and incubations with detergent, 10 μ l acetone was added. Renex 690 and unlabeled phenol were administered dissolved in 100 and 50 μ l water, respectively. The protein concentrations of the microsomal suspensions were estimated using the method of Lowry *et al.* [12].

The mono-oxygenase activity with ethoxycoumarin as substrate was measured by determination of the fluorescence of the dealkylated product 7-hydroxycoumarin [11]. The cuvette contained: 0.8 ml 0.2 M phosphate buffer pH 7.6; 1.0 ml 2×10^{-4} M ethoxycoumarin; 0.2 ml NADPH-generating system (10^{-3} M NADPH), 3×10^{-3} M glucose-6-phosphate and glucose-6-phosphate dehydrogenase (10 μ l/5 ml; Boehringer Mannheim grade II) and 0.35–0.40 mg microsomal protein. Metyrapone was added dissolved in 20 μ l water to a final concentration of 2×10^{-5} M. The detergent Renex 690 was added dissolved in 10 μ l water. The reaction was started by addition of the microsomes and was followed on a recorder for 3–4 min. During this time the rate was linear. The excitation and emission wave-lengths were 380 and 460 nm respectively. The instrument was calibrated by adding 1 μ l of a solution of 16.2 mg 7-hydroxycoumarin/100 ml water to the cuvette (1 nmole).

RESULTS

Effects of Renex 690 and metyrapone on the recovery and metabolism of phenol

To evaluate the results of this study it was necessary to investigate how Renex 690 and metyrapone affected the recovery of phenol through the extraction procedure and the further metabolism of this compound. The method used to isolate phenol has been described elsewhere, and was shown to have a recovery of 64–65 per cent [9].

Table 1 shows that Renex 690 increased the recovery of phenol from 65 to 75 per cent (due to smaller loss during the evaporation of the ethyl acetate phase). The difference of the recovery of phenol in presence or absence of Renex 690 appeared slightly (7 per cent) greater when NADPH was added to the incubation system, indicating a (very weak) inhibi-

Table 1. Effect of Renex 690 and metyrapone on the recovery and metabolism of phenol*

Modulator	Amount of phenol recovered (pmoles)	
	Incubations without NADPH-generating system	Incubations with NADPH-generating system
None	387 (65%)†	312 (52%)
Renex 690‡	450 (75%)	383 (64%)
Metyrapone‡	390 (65%)	338 (56%)

* Standard incubations were performed with 600 pmoles [14 C]phenol (35 mCi/mmole) as substrate, added dissolved in 10 μ l acetone. After incubation unmetabolized phenol was isolated. The determinations were done in duplicates which differed less than 5 per cent from each other.

† Numbers in brackets indicate percentage of total amount of phenol added to the incubations.

‡ The ratio mg Renex 690/mg microsomal protein was 0.5 and the concentration of metyrapone was 10^{-4} M.

tion of the metabolism of phenol by Renex 690. However, the overall recovery of unmetabolized phenol in incubations containing a NADPH-generating system was increased from 52 per cent to only 64 per cent. Thus, the effects of Renex 690 on the metabolism of benzene, shown in Fig. 2 and described below can only to a very minor extent be explained by effects on recovery and metabolism of phenol.

Table 1 also shows that metyrapone had no effect on extraction recovery, but a small effect on metabolism of phenol. Again the weak inhibition of phenol metabolism by metyrapone represents only a very minor contribution to the effects on benzene metabolism depicted in Fig. 1 and described below.

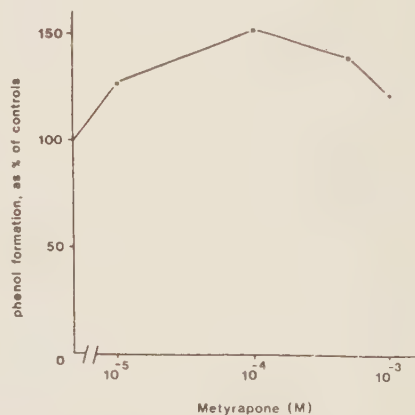


Fig. 1. The effect of metyrapone on the formation of phenol from [14 C]benzene in liver microsomes from untreated rats. The phenol formation in controls (incubations without metyrapone) was defined as 100 per cent. The specific activity in these controls was 79 pmoles phenol/mg microsomal protein/30 min. Indicated are means from two experiments, which differed by not more than 6 per cent.

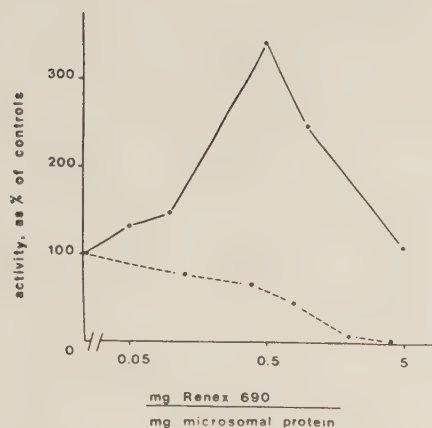


Fig. 2. The effect of the detergent Renex 690 on the formation of phenol from [^{14}C]benzene (●) and on the mono-oxygenase activity with ethoxycoumarin as substrate (*—*) in liver microsomes from untreated rats. The activities in controls (in the absence of Renex 690) are defined as 100 per cent. The specific activities in these controls were 72 pmol phenol/mg microsomal protein/30 min and 0.75 nmol 7-OH-coumarin/mg microsomal protein/min. Indicated are means from two experiments, which differed by not more than 5 per cent.

Effect of phenol on benzene metabolism

Table 2 shows the inhibition of benzene hydroxylation by phenol, the product of this hydroxylation. In presence of 50 μM or more phenol, transformation of benzene to phenol was markedly inhibited. However, the amount of [^{14}C] phenol formed from [^{14}C]benzene under the standard conditions used in this study, was around 250 pmoles, giving a phenol concentration of about 0.08 μM . In presence of 1.1 μM unlabelled phenol no measurable effect on transformation of [^{14}C]benzene to [^{14}C] phenol was observed. Thus, it seems unlikely that the amount of phenol formed under the standard conditions used would be limited by a feedback mechanism.

Table 2. Effect of unlabelled phenol on the microsomal formation of [^{14}C]phenol from [^{14}C]benzene*

Concentration of unlabelled phenol (μM)	% inhibition of [^{14}C] phenol formation
0	0
1.1	0
11	12
50	51
90	65

* Standard incubations with [^{14}C]benzene as substrate were performed. After incubation [^{14}C]phenol was isolated. In incubations without added unlabelled phenol 255 pmoles [^{14}C]phenol was formed. Unlabelled phenol was added dissolved in 50 μl of water. The determinations were made in duplicates which differed less than 8 per cent from each other.

Effects of metyrapone on phenol formation

The effects of different concentrations of metyrapone on the formation of phenol from [^{14}C]benzene in standard incubations are shown in Fig. 1. It is apparent that metyrapone, in the concentration range investigated, increased phenol formation.

Effect of detergent Renex 690 on phenol formation

In Fig. 2 the effect of the detergent Renex 690 on the phenol formation from [^{14}C]benzene in standard incubations and on the mono-oxygenase activity with ethoxycoumarin as substrate is shown. Surprisingly, the phenol formation greatly increased, while the mono-oxygenase activity measured with ethoxycoumarin was inhibited at all detergent/protein ratios investigated. At a detergent/protein ratio of 5, where mono-oxygenase activity with ethoxycoumarin as substrate was totally inhibited, the phenol formation was the same as in controls (incubations without detergent). In presence or absence of Renex 690 phenol was the only ethyl acetate-extractable metabolite of benzene formed in detectable amounts under the standard conditions of this study. This was checked using the TLC-systems described [9].

Induction of benzene and ethoxycoumarin mono-oxygenase by PB and benzene, and response of the induced activities to metyrapone and to the detergent Renex 690

Figure 3 shows that the benzene mono-oxygenase was induced about 3-fold after PB as well as after benzene treatment. As expected from the results presented in Figs. 1 and 2, both metyrapone and Renex 690 stimulated the benzene mono-oxygenase activity in control microsomes. However, the PB-induced activity was inhibited by metyrapone down to the level observed in metyrapone-treated controls. Also, in the presence of Renex 690 the activity was the same as in Renex 690-treated controls. The benzene-induced activity, on the other hand, was stimulated by metyrapone and Renex 690, as was the activity in controls.

In Fig. 4 data on mono-oxygenase activity with ethoxycoumarin as substrate are shown. Both PB and benzene induced the activity about 3-fold. The controls and benzene-induced activities were inhibited by 30–40 per cent by metyrapone as well as Renex 690. The PB-induced activity, on the other hand, was inhibited by about 80 per cent by the two modulators.

DISCUSSION

Metyrapone is known to be an inhibitor of several mono-oxygenase forms (see e.g. [13]). The activation of benzene mono-oxygenase by metyrapone in control and in benzene-induced microsomes reported in this paper stands in contrast to these inhibitory effects but is comparable to the activation [14, 15] of acetanilide hydroxylation by at least some metyrapone concentrations. Gonasun *et al.* [10] investigated the effect of metyrapone on phenol formation from benzene in mouse liver microsomes. At a concentration of 10^{-4} M, where we found 50 per cent activation in the rat, they reported a weak inhibition

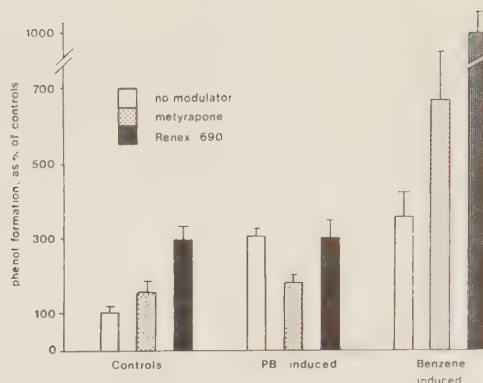


Fig. 3. Induction of benzene hydroxylase with PB and benzene and the effects on the induced and control activities of Renex 690 and metyrapone. Control: The activity in microsomes from untreated rats incubated without metyrapone or detergent. The specific activity in these controls was defined as 100 per cent and was 77 pmole phenol/mg microsomal protein/30 min. Metyrapone was used at a concentration of 5×10^{-4} M and the ratio mg Renex 690/mg microsomal protein was 0.5. Each bar represents the mean of independent measurements from three animals, and the standard deviations are indicated.

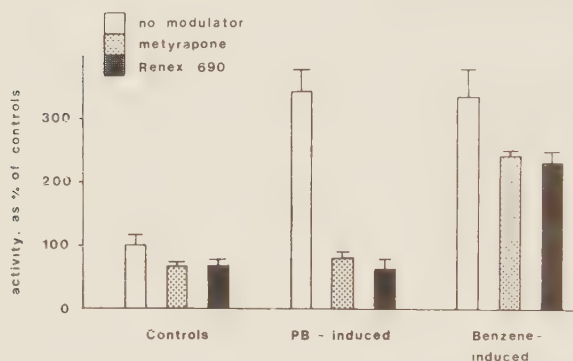


Fig. 4. Induction of mono-oxygenase activity, as measured with the ethoxycoumarin assay, with PB and benzene, and the effects, on the induced and control activities, of metyrapone and the detergent Renex 690. The control activity was 0.69 nmole/mg microsomal protein/min. Metyrapone was used at a concentration of 2×10^{-3} M. See also legend to Fig. 3.

(20 per cent). To decide if this discrepancy reflects different benzene mono-oxygenase enzymes in the two species, or if it is due to different experimental design, requires further investigation.

The activities of some non-oxidative microsomal enzymes are increased by detergent treatment [16-19]. If an ordered spatial arrangement between NADPH cytochrome P-450 reductase and cytochrome P-450 (cf. [20]) is important for mono-oxygenase activity, detergent treatment may decrease mono-oxygenase activity by loosening such an ordered structure. Moreover, it has been shown that detergents accelerate the denaturation of cyto-

chrome P-450 to the metabolically inactive P-420 form [21]. Thus it was as expected when we found that addition of detergent inhibited the mono-oxygenase activity as measured with the ethoxycoumarin assay (Fig. 2). This assay is known to measure a very wide spectrum of mono-oxygenases [13]. To our surprise, however, we found a massive activation of the benzene mono-oxygenase activity on addition of the detergent (Fig. 2). At a detergent concentration, where the mono-oxygenase activity with ethoxycoumarin as substrate was totally inhibited, the benzene mono-oxygenase activity was the same as without the detergent. To the best of our knowledge this

represents the first demonstration of an activation of microsomal mono-oxygenase by detergent treatment.

Since the concentration of benzene in the incubation mixtures (7 μ M) was far below the solubility limit (11 mM), we see two possible explanations for the activation by the detergent:

1. Although the results in Table 2 indicate that the amounts of phenol formed are much too low to inhibit benzene hydroxylation, it could still be that newly formed phenol reaches high local concentrations at its site of formation, i.e. at the cytochrome P-450, concentrations high enough to compete with unmetabolized benzene. In this case the detergent may increase the dissociation of the phenol from the cytochrome.
2. A special form of mono-oxygenase, with the unusual property of being activated by detergent, might be responsible for the benzene mono-oxygenase activity.

The results given in Fig. 3 favour the latter explanation. It is unlikely that some special property of the substrate benzene, or of the product phenol, would be responsible for the unusual effects of detergent and metyrapone on the benzene hydroxylation since these effects are not seen in PB-induced microsomes. The most obvious explanation of these findings would be instead that (1) there exists already in control microsomes an unknown number of mono-oxygenase forms which accept benzene as substrate and which are activated by metyrapone and by detergent; (2) PB induces a mono-oxygenase form or forms which can also accept benzene as substrate but which are basically different from the control benzene mono-oxygenase(s) in that they are not activated by either metyrapone or by detergent; (3) benzene induces the benzene mono-oxygenase form(s) which are already present in control microsomes.

The properties of induced mono-oxygenase activity, as measured with the ethoxycoumarin assay, are shown in Fig. 4. Benzene- and PB-treatment both induced the activity about 3-fold. The PB-induced activity was massively inhibited by metyrapone and detergent while the activity in controls and in microsomes from benzene-induced rats was inhibited by only 30–40 per cent.

Thus, whether assayed with benzene or 7-ethoxycoumarin as substrates, and Renex 690 or metyrapone as modulators, the benzene-induced mono-oxygenase activity possesses characteristics resembling the controls rather than that induced by phen-

obarbital. This observation is of interest since it is often assumed that PB induces mainly the control forms of cytochrome P-450 or forms having properties similar to the control forms. Thus it seems important for future studies to investigate the properties of the benzene-induced mono-oxygenase activity further, since the induction of a mono-oxygenase activity, in substrate specificity and cytochrome P-450 composition as much as possible resembling the control forms would be of great interest.

Acknowledgements—This work was supported by the Swedish Cancer Society and the Stiftung Volkswagenwerk.

REFERENCES

1. M. Berlin, J. C. Gage and E. Jonsson, *Work-environment* **11**, 1 (1974).
2. R. Snyder and J. J. Kocsis, *CRC Crit. Rev. Toxicol.* **3**, 265 (1975).
3. R. Snyder, E. W. Lee, J. J. Kocsis and C. M. Witmer, *Life Sci.* **21**, 1709 (1977).
4. L. S. Andrews, E. W. Lee, C. M. Witmer, J. J. Kocsis and R. Snyder, *Biochem. Pharmac.* **26**, 293 (1977).
5. F. Oesch, *Xenobiotica* **3**, 305 (1973).
6. D. Jerina and J. W. Daly, *Science* **185**, 573 (1974).
7. P. Sims and P. L. Grover, *Adv. Cancer Res.* **20**, 165 (1974).
8. C. Heidelberger, *A. Rev. Biochem.* **44**, 79 (1975).
9. A. Tunek, K. Platt, P. Bentley and F. Oesch, *Molec. Pharmac.* **14**, 920 (1978).
10. L. M. Gonasun, C. M. Witmer, J. J. Kocsis and R. Snyder, *Toxicol. appl. Pharmac.* **26**, 398 (1973).
11. V. Ullrich and P. Weber, *Hoppe-Seyler's Z. physiol. Chem.* **353**, 1171 (1972).
12. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
13. V. Ullrich, P. Weber and P. Wollenberg, *Biochem. Biophys. Res. Commun.* **64**, 808 (1975).
14. K. C. Leibman, *Molec. Pharmac.* **5**, 1 (1969).
15. G. F. Kahl, K. Minck and K. J. Netter, *Drug. Metab. Dispos.* **1**, 191 (1973).
16. A. Winsnes, *Biochim. biophys. Acta* **191**, 279 (1969).
17. R. D. Howland, A. Burkhalter, A. J. Trevor, S. Hegeman and D. V. Shirachi, *Biochem. J.* **125**, 991 (1971).
18. R. C. Nordlie, in *Current Topics in Cellular Regulation* (Eds. B. L. Horecker and E. R. Stadtman), Vol. 8, p. 33. Academic Press, New York (1974).
19. B. Burchell, P. Bentley and F. Oesch, *Biochim. biophys. Acta* **444**, 531 (1976).
20. J. A. Peterson, R. E. Ebel, D. H. O'Keffe, T. Matsubara and R. W. Estabrook, *J. biol. Chem.* **251**, 4010 (1976).
21. A. Y. H. Lu and W. Levin, *Biochim. biophys. Acta* **344**, 205 (1974).

Microsomal Metabolism of Benzene to Species Irreversibly Binding to Microsomal Protein and Effects of Modifications of This Metabolism

ANDERS TUNEK,¹ KARL L. PLATT, PHILIP BENTLEY, AND FRANZ OESCH

Section on Biochemical Pharmacology, Institute of Pharmacology, University, Obere Zahlbacher Straße 67, D-6500 Mainz, West-Germany

(Received February 2, 1978)

(Accepted April 3, 1978)

SUMMARY

TUNEK, ANDERS, PLATT, KARL, BENTLEY, PHILIP & OESCH, FRANZ (1978) Microsomal metabolism of benzene to species irreversibly binding to microsomal protein and effects of modifications of this metabolism. *Mol. Pharmacol.*, 14, 920-929.

It has been shown that when [¹⁴C]benzene is incubated with rat liver microsomes in the presence of a NADPH-generating system, metabolites which irreversibly bind to bio-macromolecules are formed. This binding occurs mainly to microsomal protein rather than ribonucleic acids. The addition of 2 mM reduced glutathione or cysteine to the incubation mixture prevented 90-95% of the binding but had only a small effect on the formation of phenol, the main metabolite of benzene and rearrangement product of the putative reactive intermediate benzene oxide. The rate of phenol formation was approximately the same when fully deuterated benzene and normal benzene were used as substrates. This is compatible with phenol formation via benzene oxide and not possible if the C-H cleaving of a direct insertion reaction were the rate-limiting step. The decrease in binding in presence of reduced glutathione was accompanied by a corresponding increase in the amount of water soluble metabolites. In contrast to the situation with metabolically activated benzene, the putative reactive metabolite benzene oxide led to negligible binding and reduced glutathione had no effect on the formation of water soluble metabolites, i.e., the spontaneous conjugation of benzene oxide and reduced glutathione was practically zero at the physiological pH of the experiments. However, when [³H]-phenol was incubated with rat liver microsomes and a NADPH-generating system, effects similar to those with benzene were observed in that marked irreversible binding occurred and this binding was prevented by reduced glutathione. The extent of binding was much greater when using phenol. These results are compatible with the assumption that an immediate metabolite of phenol rather than of benzene is responsible for the binding. This assumption is supported by the fact that addition of uridine-diphosphate-glucuronic acid to incubations with [¹⁴C]benzene decreased the amount of both phenol and the irreversibly bound metabolites. Metabolism of benzene to 1,2-dihydro-1,2-dihydroxybenzene was observable, but only when pure epoxide hydratase was added in amounts greatly exceeding those already present in the microsomes. Increased formation of this diol upon addition of homogeneous epoxide hydratase is unequivocal proof for the metabolic

This work was supported by Deutscher Akademischer Austauschdienst, the Swedish Cancer Society (A. T.) and the Stiftung Volkswagenwerk.

¹ Recipient of a fellowship from Deutscher Akademischer Austauschdienst and a travel award from

Riksföreningen mot Cancer (Swedish Cancer Society). Permanent address: Department of Environmental Health, University of Lund, Sölvegatan 21, S-223 62 Lund, Sweden.

formation of benzene oxide, which has so far eluded isolation. The observations (i) prove that reactive metabolite(s) which irreversibly bind to microsomal protein are formed during microsomal metabolism of benzene and that this binding is prevented by low molecular weight nucleophiles such as glutathione or cysteine; (ii) exclude benzene oxide as the metabolite predominantly responsible for this binding; and (iii) strongly indicate that a secondary metabolite of benzene, namely a metabolite of phenol, is mainly responsible for this binding.

INTRODUCTION

Benzene is one of the most widely distributed environmental pollutants, mainly because it is a constituent of most motor fuels. For many decades, it has been known that chronic exposure to benzene may lead to bone marrow damage. The current concepts of benzene toxicology and metabolism have recently been thoroughly reviewed (1).

It has been shown that benzene is oxidized by a microsomal cytochrome P-450-dependent monooxygenase (2). The principal metabolite is phenol, which is excreted via the urine either in the free form or as sulfate and/or glucuronic acid conjugates (3, 4). *In vivo* a number of other metabolites have been detected in small quantities: CO₂, hydroquinone, catechol, hydroxyhydroquinone, *trans-trans*-muconic acid, L-phenyl mercapturic acid (3, 5) and benzene dihydrodiol² (6). In experiments with isolated microsomes, only phenol (2, 7) and to a small extent benzyl alcohol (8) have been reported as metabolites of benzene.

Some evidence exists to support the idea that a metabolite of benzene is responsible for the adverse effects of benzene on bone marrow (9). Polycyclic aromatic hydrocarbons have been shown to be metabolically oxidized via epoxides, and epoxides are believed to be the ultimate toxic and carcinogenic derivatives of these compounds (10-14). In analogy, benzene oxide has been suspected to be the ultimate toxic agent of benzene. This concept was supported by the finding that *in vitro* metabolism of benzene oxide gave the same main groups of metabolites as the *in vivo* metabolism of benzene (15). If benzene were metabolized

via an electrophilic intermediate, e. g., an epoxide, it would be expected to bind covalently to nucleophilic centers in biomacromolecules. Protection against this binding by competing nucleophiles such as GSH² would be expected. If so, it would also be expected that changes in the concentration of GSH would lead to changes in the pattern of benzene metabolites. Reaction of GSH with a number of electrophilic agents (16) has been reported. Of special interest is that it has been shown to react with benzene oxide enzymatically and also to a certain extent spontaneously (15). However, attempts to prove metabolism of benzene to benzene oxide using radiotracer trapping technique were unsuccessful (15).

In this study we have investigated the possibility that benzene metabolites become irreversibly bound to microsomal macromolecules during metabolism of benzene by rat liver microsomes and the influence of modulators of metabolism on the distribution of benzene metabolites between irreversibly bound, water soluble and ethyl acetate-soluble metabolites. Our findings show that benzene metabolites do bind irreversibly to microsomal proteins during incubation in the presence of a NADPH-generating system, exclude benzene oxide as the metabolite predominantly responsible for this binding and indicate that the metabolite that causes the main portion of the binding is a secondary metabolite of benzene, namely a metabolite of phenol.

MATERIALS AND METHODS

Compounds. All chemicals, obtained from commercial sources, were of analytical grade. [¹⁴C]Benzene from the Radiochemical Centre, Amersham, had a specific activity of 107 mCi/mmol, and was >99% radiochemically pure. The standards needed for the recovery study were obtained from benzene oxide-[3,6-³H] which was synthesized

² Abbreviations used are: benzene dihydrodiol, *trans*-1,2-dihydro-1,2-dihydroxy benzene; GSH, reduced glutathione; TCA, trichloroacetic acid; UDPGA, uridine-diphosphate-glucuronic acid.

as described (17) and had a specific radioactivity of 0.08 mCi/mmol.

[^3H]phenol was prepared by adding about 2×10^6 dpm [^3H]benzene oxide dissolved in 25 μl acetonitrile, to 1 ml 5 M HCl. After two extractions with 4 ml ethyl acetate, the pooled organic phases were dried over Na_2SO_4 and evaporated. The residue was dissolved in 200 μl ethyl acetate and applied as a band to a thin layer plate which was developed in system 1 (see below) together with unlabeled commercial phenol as a reference. The phenol band was scraped off and eluted with ethyl acetate. After filtration and evaporation the remainder was dissolved in acetone to give 1.44×10^5 dpm/10 μl . The identity of the [^3H]phenol was confirmed by thin layer chromatography in system 2 and 3 (see below) and the purity was found to be >99%.

[^3H]benzene dihydrodiol was prepared by incubating overnight at 5° C approximately 6×10^6 dpm [^3H]benzene oxide with rat liver microsomes (3 mg protein) in 2 ml 0.5 M tris buffer pH 9.0 containing 1.25% acetonitrile. After incubation the [^3H]benzene dihydrodiol was isolated by thin layer chromatography as described above for [^3H]phenol but using ethyl acetate as solvent. Unlabeled benzene dihydrodiol used as reference was synthesized as described (18). The product was dissolved in acetone to give 27,000 dpm/10 μl . The identity of the diol was confirmed by thin layer chromatography in system 3 (see below) and the purity was found to be >97%.

Thin layer chromatography. Thin layer chromatography was carried out on pre-coated glass plates, silica gel, 60 F-254, Merck. The plates were developed with benzene:chloroform:ethyl acetate (1:1:1) (system 1), ethyl acetate (system 2) or benzene:dioxane:acetic acid (19:5:1) (system 3). Phenol had R_f values of 0.48, 0.65, and 0.60, respectively and benzene-dihydrodiol 0, 0.27 and 0.21, respectively, in the three systems.

Preparation of microsomes. Male Sprague-Dawley rats, weighing about 250 g, were killed by decapitation. The livers were removed, washed in ice cold 50 mM tris buffer, pH 7.5, containing 0.25 M sucrose and homogenized in a Potter-Elvehjem homogenizer in four volumes of the

same buffer. This homogenate was centrifuged for 10 min at 10,000 g , and the supernatant fraction centrifuged for 60 min at 105,000 g . These pellets were suspended in 0.15 M KCl, 4 ml/g liver wet weight, and centrifuged for 30 min at 105,000 g . The pellets were then suspended in 50 mM tris buffer, pH 7.5 containing 0.25 M sucrose, to yield a protein concentration of 15 mg/ml, estimated using the method of Lowry *et al.* (19) with bovine serum albumin as standard.

Incubations. The standard incubations contained: 1.5 ml of buffer A (100 mM tris pH 7.5, 10 mM MgCl_2 and 10 μM MnCl_2), a NADPH-generating system (3 mg glucose-6-phosphate, 2 mg NADP and 4 μg (0.56 U) glucose-6-phosphate dehydrogenase (Boehringer, Mannheim)) and 0.2 ml microsomal suspension (3 mg protein) in a total volume of 3 ml. The substrates, 21.5 nmoles [^{14}C]benzene, 812 nmoles [^3H]phenol or 14.1 μmoles [^3H]benzene oxide were added dissolved in 10 μl acetone. Incubations were started by adding the substrate to incubation mixtures which had been preincubated for 2 min. All incubations were performed at 37°C in stoppered glass tubes. Incubation time was 1 hr, unless otherwise stated.

In two series of experiments the standard incubations were modified: When epoxide hydratase, purified as described (20), was added to the incubation mixtures, these were reduced to one sixth the volume described above in order to reduce the amount of enzyme needed. The epoxide hydratase was added in 100 μl of buffer A diluted with one volume of water. One hundred microliters of enzyme-free diluted buffer A was added to the controls. Substrate [^{14}C]benzene (4.0 nmoles) was added in 2 μl acetone. After stopping the incubations as described below, 0.2 ml heat-denatured microsomal suspension and 2.0 ml buffer A diluted with one volume of water was added in order to reduce the relative loss of protein during the wash and extraction procedure described below.

The second modification of the incubation mixtures was in the determination of isotope effect on the phenol formation, where the amounts and volumes of the standard incubations were doubled and the

incubation time was reduced to 30 min. One μ l of normal or of fully deuteriated benzene dissolved in 9 μ l acetone was added as substrate. Thus the substrate concentration in this experiment was 2.0 mM.

Separation of metabolites into different groups. The incubations were stopped by addition of 5 ml ethyl acetate and shaking on a rotoshake (Kühner AG, Basel) for 5 min at 40 rpm. The samples were then centrifuged at 1800 *g* for 10 min, and the organic phases transferred to other tubes. The ethyl acetate extraction was repeated twice more and the organic phases from each sample were combined and treated as described below. Three ml ethanol were added to the water phases to precipitate the proteins and the samples were vigorously shaken and centrifuged for 15 min at 1800 *g*. One ml of the resulting supernatant fraction was then added to 10 ml Unisolve (Koch-Light Laboratories, Colnbrooks Bucks, England) followed by determination of radioactivity by scintillation spectrometry. This fraction is termed water-soluble metabolites.

The remainder of the water-ethanol supernatant fraction was decanted off, and the precipitated microsomal pellets were washed with 5 ml acetone/hexane (25:10) and 5 ml methanol, by shaking on a Vortex shaker with a stainless-steel spatula inserted in the tube to facilitate mixing as described by Jollow *et al.* (21). The pellets were then dissolved in 2 ml 1 M NaOH at 80°C for 30 min. After neutralization with 2 ml 1 M HCl, an aliquot of 1 ml was added to 10 ml Unisolve and the radioactivity estimated by scintillation spectrometry. This fraction is called irreversibly bound metabolites.

The irreversibly bound nature of these metabolites was checked by further washing the pellets with hexane, chloroform, ethyl ether, acetone, ethanol, methanol and 10% TCA². Our method of preparing irreversibly bound metabolites was also compared with that described by Jollow *et al.* (21).

The ethyl acetate phases were evaporated to dryness on a rotation evaporator. This process was carefully observed and stopped as soon as all the solvent had evaporated. The remainder was dissolved in 1

ml ethyl acetate. An aliquot of 200 μ l was then applied as a band on thin layer chromatography plates using unlabeled phenol and/or benzene dihydrodiol as reference. The plates were developed in one of the three solvent systems described above, and in each experiment the identity of the metabolites in one incubation mixture was confirmed by using also the other two systems. The phenol and benzene-dihydrodiol bands were scraped off and the silica gel added to 6 ml 0.6% Butyl-PBD (Koch Light Laboratories, Colnbrooks Bucks, England) and counted for radioactivity after standing 15 hr in a refrigerator.

In the study of the isotope effect on phenol formation, a different method was used to quantitate phenol. The incubation mixtures, modified as described above, were extracted three times with 10 ml ethyl acetate and the organic phases thereafter evaporated to dryness. The residue was dissolved in 100 μ l acetone and 2 μ l was injected into a gas chromatograph (Packard model 427) using a column of 2.5% SE 52 on 80–120 mesh Chromosorb W and a flame ionization detector. The carrier gas was nitrogen and the temperatures of the column, the injector and the detector were 70°, 170° and 250° C, respectively. The area of the phenol peak was determined using an integrator (Packard model 603).

RNase and protease treatment. Standard incubations were performed as described. The incubations were stopped by boiling for 5 min. After cooling, the microsomes were rehomogenized and treated for 24 hr at 37°C with 6000 units Ribonuclease T₁ (Boehringer Mannheim) or 80 units Protease Type VI (Sigma), dissolved in 100 μ l buffer A diluted with one volume of water. Irreversibly bound metabolites were then separated from radioactivity associated with low molecular weight material as described above.

Recovery studies. Recovery studies were performed for [³H]phenol and [³H]benzene dihydrodiol in the above described extraction scheme. Standard incubations lacking the NADPH-generating system were performed with 10 μ l of the acetone solutions described under Compounds.

Calibration. The results from the scintillation spectrometry were transformed to

dpm by the use of the external standard of the instrument. This procedure was occasionally checked by the use of [^{14}C]toluene, [^3H]toluene or [^3H]water as an internal standard. In all experiments zero time incubations were run, and the results from these were subtracted from all other results, except in Figs. 2 and 4 where results are compared to such blanks.

Since the ortho-tritium of the 2,5-ditritio-phenol used may be exchanged, the tritium loss during the standard incubation was monitored. This loss was only 5–9% and the results are not corrected for this loss. Molarities and specific radioactivities given refer to amount of phenol determined spectrophotometrically and the radioactivity determined by scintillation spectrometry.

RESULTS

Recovery study. The recovery of phenol and benzene dihydrodiol was estimated by extracting the [^3H]-labeled compounds in the normal manner from incubation mixtures lacking a NADPH-generating system. The recovery of phenol from the thin-layer chromatography plates was $64 \pm 5\%$ ($n = 5$). Phenol contamination of the water phase and the irreversibly bound metabolites were estimated to be $3 \pm 1\%$ and $2 \pm 1\%$, respectively. The recovery of benzene dihydrodiol was $50\% \pm 5\%$ ($n = 3$). Benzene dihydrodiol did not contaminate the irreversibly bound fraction, but 15% of the compound was found in the water phase. However, as shown below, benzene dihydrodiol was not formed under standard conditions and thus this possible contamination of the water phase was unimportant for the purposes of this study.

Time course studies. The results of the time course studies are shown in Fig. 1. The formation of water soluble and irreversibly bound metabolites was practically linear for at least 30 min, while phenol formation was only linear for 10 min. GSH inhibited the formation of irreversibly bound metabolites, and increased the production of water soluble compounds about fourfold. The phenol formation was less affected. The sum of recovered metabolites in the presence of GSH was 81% of the sum in the absence of this cofactor, reflecting either a slight inhibition of the metabolism or a

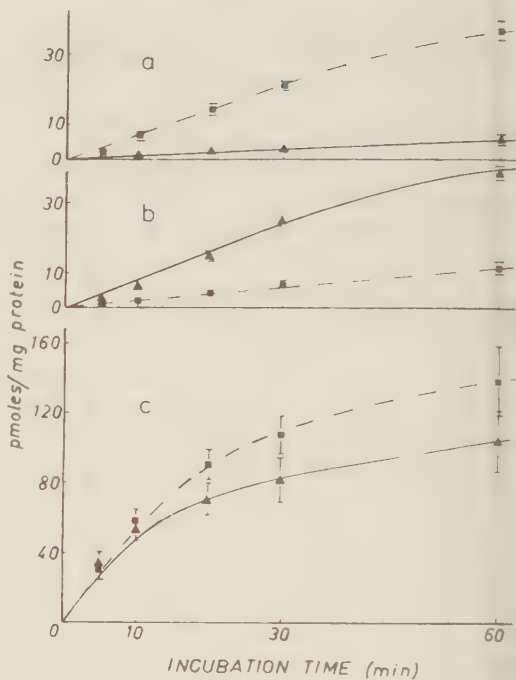


FIG. 1. Formation of microsomal metabolites derived from benzene as a function of the incubation time

a. Metabolites irreversibly bound to microsomes, b. water soluble metabolites, c. phenol. Incubations were performed in the absence (—■—) or presence (—▲—) of GSH (2 mM). All values are based on three determinations, and zero time results have been subtracted. Except for the irreversibly bound metabolites in the presence of GSH and the water soluble metabolites without GSH, the radioactivity recovered in the different phases from zero time incubations, as well as from 1 hr-incubations lacking a NADPH-generating system, was always less than 5% of the radioactivity recovered from the standard 1 hr-incubations.

slight deviation to more volatile metabolites.

After 1 hr incubation in the absence of GSH 555 pmoles metabolites were formed per incubation mixture (3 mg protein), i.e., 2.6% of the [^{14}C]benzene had been metabolized. Of these metabolites 20% became irreversibly bound to the macromolecules. This time point was chosen for the further studies described in this paper.

We were unable to show the formation of benzene dihydrodiol. If at all formed under normal conditions, the diol amounts to less than 1% of the amount of phenol, i.e., less than 0.03% of the incubated benzene. Phenol always accounted for at least 95%

of the radioactivity above background recovered from the thin layer plates.

Further studies on the irreversible binding. In order to gain some insight into the characteristics of the binding of benzene metabolites the experiments summarized in Fig. 2 were performed.

The method of washing the microsomes to remove free and reversibly bound metabolites described by Jollow *et al.* (21) gave 20% higher values of the binding (II) than our method (I). Further washing after our standard washing procedure did not decrease the binding to any significant degree (V). The radioactivity in the fraction of irreversibly bound metabolites was not significantly higher than zero time incubations (III) after incubation with boiled microsomes (XI) or with active microsomes without NADPH (IV). After protease treatment

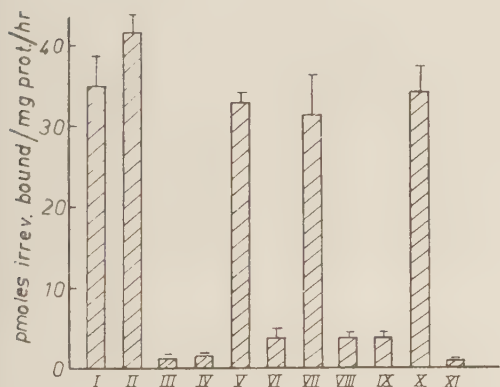


FIG. 2. Some studies on the irreversibly bound metabolites

Standard incubations with [^{14}C]benzene as described under MATERIALS AND METHODS. I. Irreversibly bound fraction when washed as described in MATERIALS AND METHODS; II. irreversibly bound fraction when washed as described by Jollow *et al.* (21); III. zero-time incubations; IV. 1-hr-incubations lacking a NADPH-generating system; V. effect of further washes with hexane, chloroform, ethyl ether, acetone, ethanol, methanol and 10% TCA as described; VI. effect of protease treatment; VII. effect of RNase treatment; VIII. effect of 2 mM GSH; IX. effect of 2 mM cysteine; X. effect of 2 mM GSH added 5 min before the end of incubation; and XI. incubation with boiled microsomes. I and II were determined in 5 experiments and standard deviations are indicated. III-XI were performed duplicates and the ranges are indicated. Zero-time incubations (III) have been subtracted as blank values from all values reported in this paper except those of this figure and figure 4.

only 10% of the binding remained (VI), while RNase treatment had little effect (VII). Both GSH (VIII) and cysteine (IX) inhibited the binding by about 90%. GSH added 5 min before the end of 1 hr incubation had no detectable effect (X), indicating that GSH did not cleave the bound material but prevented the binding.

Effects of different concentrations of GSH on the irreversible binding and phenol formation. The effect of different concentrations of GSH on phenol formation and on the irreversible binding in standard incubations is shown in Fig. 3. While the inhibition of the binding is dose dependent reaching 95% inhibition, the effect on phenol formation is weak, the ranges reaching maximally 30% inhibition. Between 500 and 5000 μM GSH, where inhibition of binding is still strictly dose-dependent, no further inhibition of phenol formation was visible. The increase in the amount of water soluble metabolites approximately corresponded to the decrease in binding (data from 0-1 mM not shown; for a concentration of 2 mM GSH see Fig. 1).

Determination of the isotope effect on the rate of phenol formation. Incubations were performed as described and phenol was isolated and analyzed by gas chromatography. Using normal benzene the rate of phenol formation was 13.2 ± 0.84 nmole/30 min ($n = 5$) while from fully deuterated benzene the rate of phenol formation was 12.0 ± 1.16 nmole/30 min ($n = 5$). Thus $K_H/K_D = 1.10 \pm 0.13$, which shows that the rate of hydroxylation of benzene to phenol is not subject to a significant isotope effect.

Determination of irreversible binding and spontaneous GSH conjugation of [^3H]benzene oxide. When standard incubations lacking a NADPH generating system were performed using [^3H]benzene oxide as substrate very little radioactivity was recovered in the irreversibly bound fraction (0.3-0.4%). Addition of 2 mM GSH to the incubation mixtures had no effect on this binding. The amount of radioactivity recovered in the water phase was 2-3%. This was also unaffected by addition of GSH.

Incubations with [^3H]phenol. Figure 4 shows that metabolites which could bind irreversibly to macromolecules were formed from [^3H]phenol and that this bind-

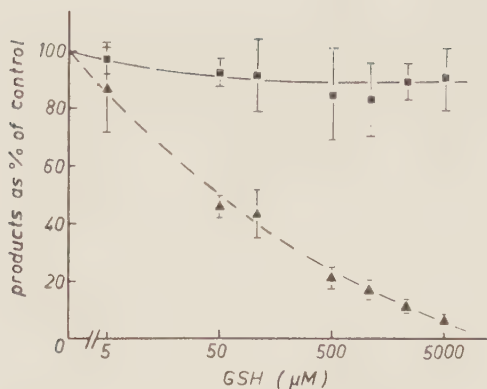


FIG. 3. Formation of phenol (■—■) and irreversible binding (▲—▲) at different concentrations of GSH

Standard incubations with [14 C]benzene and isolation of metabolites were performed as described in MATERIALS AND METHODS. As controls standard incubations without GSH were used. The amount of products formed in these was defined as 100%. Means and standard deviations of four experiments are shown.

ing was prevented by GSH. However, while only 0.6% of the administered dose of benzene bound as shown above, almost 10% of the phenol dose administered in this experiment bound, although about 40-fold more phenol than benzene was given.

Incubations with microsomes and purified epoxide hydratase. Incubations were performed in the presence of different amounts of epoxide hydratase. As stated above we could not show the formation of benzene-dihydrodiol during standard incubation conditions. Fig. 5 shows that the diol could be detected if relatively large amounts of purified epoxide hydratase were added.³ At the highest amount of epoxide hydratase the formation of diol was about 20% of the phenol formation. This large amount of purified epoxide hydratase (about 40-fold that present in the microsomes used for this experiment) inhibited the irreversible binding during benzene metabolism by about 40%. This does not reflect epoxide hydratase activity, however,

³ The point of the graph at 0 units epoxide hydratase added (i. e. in presence of the 3-4 units present in the microsomes used) represents the duplicate sample of the individual experiment shown. However, many (>10) similar experiment showed no or similar trace amounts of diol.

since the same degree of inhibition was observed using the same amount of boiled epoxide hydratase. Destruction of enzyme activity during boiling was proven by absence of diol formation. It is unlikely that free sulphhydryl groups of the denatured

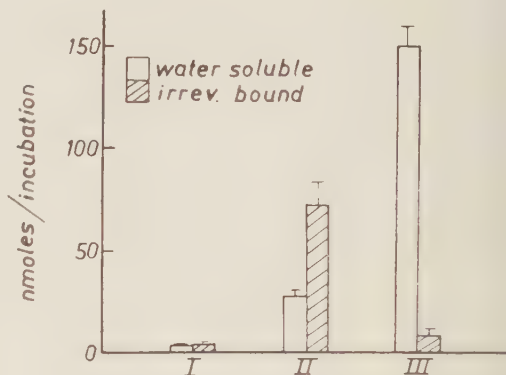


FIG. 4. Formation of water soluble and irreversibly bound metabolites from [3 H]phenol and the dependence of this formation on the NADPH-generating system and GSH

I. Standard incubations lacking a NADPH-generating system, II. standard incubations and III. standard incubations with 2 mM GSH. As substrate 812 nmoles [3 H]phenol was used. Two experiments were performed and means and ranges are indicated.

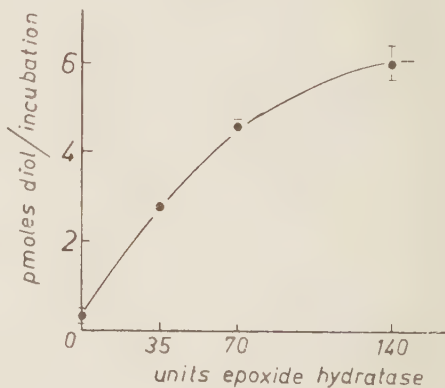


FIG. 5. Formation of benzene-dihydrodiol on addition of purified epoxide hydratase

Isolation and identification of the diol and modification of incubation mixtures are described in MATERIALS AND METHODS. In incubations containing the largest amount of hydratase, the amount of diol measured by scintillation spectrometry, was 6-fold higher than in blank incubations lacking the NADPH-generating system or zero-time incubation. Incubations were performed in duplicate, and means and ranges are indicated.

enzyme are responsible for the inhibition of binding, since bovine serum albumin (up to twice the amount of epoxide hydratase used) did not show this effect.

Effect of UDPGA². Standard incubations using [¹⁴C]benzene as substrate and with concentrations of UDPGA ranging between 0 and 30 mM, were performed. The results for phenol and irreversibly bound metabolites are shown in Fig. 6. As expected, UDPGA lowered the amount of phenol recovered. However, the effect on the irreversible binding was even stronger. The formation of water soluble metabolites increased on addition of UDPGA in such a way that the total metabolism up to 10 mM UDPGA concentration was constant or slightly (~10%) elevated. At 30 mM UDPGA, however, the total metabolism was only 80% of that observed without the cofactor.

DISCUSSION

The method of detecting irreversibly bound metabolites, developed for the purpose of this study, enabled us to measure the formation of phenol, benzene dihydrodiol, water soluble metabolites and irreversibly bound metabolites in the same incubation mixture. Although we have not

isolated and identified any water soluble metabolites in this study, we felt it was essential to determine their total amount, in order to see whether the different modifications to the incubation conditions simply inhibited (or activated) benzene metabolism. The results of the recovery study show that the possible contaminating compounds phenol and benzene dihydrodiol do not significantly contaminate the irreversibly bound fraction. From Fig. 2 it is clear that the contamination by unmetabolized benzene is quite small (III, IV and XI), that the irreversible binding occurs to proteins rather than to ribosomal RNA (VI and VII) and that nucleophilic agents such as GSH and cysteine effectively inhibit the binding (VIII and IX).

These findings are consistent with the hypothesis that benzene is hydroxylated via an epoxide intermediate and that this intermediate is responsible for the binding. The results presented in Fig. 3, however, are difficult to explain on the basis of this hypothesis. The effect of GSH on phenol formation is very weak (<20% inhibition, the ranges of the effects reaching maximally 30%) and the effect does not increase significantly between 50 μ M and 5 mM. The effect of GSH on the binding at these concentrations is, however, clearly dose-dependent and reaches 95% inhibition at 5 mM.

We therefore decided to investigate whether phenol was formed via a route which did not involve any electrophilic intermediates, e.g., by direct insertion. The rate of hydroxylations that proceed via direct insertion show an isotope effect (K_H/K_D) of 1.3–1.7 (except where a step other than C-H cleaving is rate limiting, e.g., binding of the substrate to the enzyme), while the rate of hydroxylation via epoxide formation followed by NIH-shift shows no isotope effect (22). Thus, since no isotope effect was found, phenol is most probably formed (at least predominately) via the arene oxide route (23). This is further supported by the findings presented in Fig. 5. It is shown that benzene dihydrodiol is formed upon addition of epoxide hydratase to incubation mixtures with [¹⁴C]benzene as substrate. The activity of epoxide hydratase occurring in rat liver microsomes

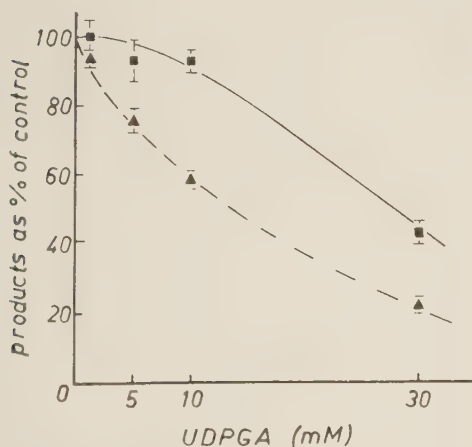


FIG. 6. The effect of UDPGA on the amount of phenol (■—■) and irreversibly bound metabolites (▲---▲)

Standard incubations with [¹⁴C]benzene as substrate were performed in duplicate and means and ranges are indicated. As controls standard incubations without UDPGA were used. The amount of products formed in these was defined as 100%.

is 7-9 units⁴/mg protein (20). Thus 3-4 units were present in the microsomes used in these modified incubations, while 35-104 units purified enzyme were added in the experiment depicted in Fig. 5. This need for high epoxide hydratase addition agrees with the instability of benzene oxide and its high apparent K_m (about 30 mM) in the hydration reaction.⁵

Traces of benzene dihydrodiol have been reported to occur in rabbit urine after a heavy dose of benzene (6). It has also been suggested that the catechol excreted by rabbits after dosage with benzene possibly originates from a dihydrodiol (3, 5), although in these studies the diol itself could not be detected. However, since these were studies in whole animals, a contribution from intestinal bacteria is always possible and multistep reactions leading to dihydrodiol not via an epoxide can less readily be excluded than in the presence of liver microsomal fraction. The findings presented in this study constitute the first evidence that benzene dihydrodiol is formed from benzene *in vitro*. This in combination with the fact that benzene was converted to observable quantities of benzene dihydrodiol only after addition of homogeneous epoxide hydratase constitutes unequivocal proof of the metabolic formation of benzene oxide. Moreover, the fact that the diol formation, in the presence of large amounts of epoxide hydratase, is about 20% of the phenol formation shows that a considerable portion of benzene hydroxylation proceeds via the epoxide intermediate.

Benzene oxide, when incubated with microsomes under standard conditions, did not (or at most minimally) bind irreversibly to macromolecules, nor did it form water soluble conjugates with GSH. These observations are in contrast to those when benzene was used as substrate. Experiments with benzene oxide should always be interpreted with caution, due to the instability of the compound. However, the half-life of benzene oxide in water pH 7.0 is estimated to be about 2 min (11), and since the bind-

⁴ One unit epoxide hydratase is defined as the amount of enzyme producing 1 nmole styrene glycol per min from styrene oxide (26).

⁵ M. Golan, P. Bentley, K. Platt and F. Oesch, publication in progress.

ing of an electrophilically reactive metabolite can be expected to proceed non-enzymically, a proportionality with respect to the concentration of the reactive species may be assumed. Thus, these observations exclude benzene oxide as the agent predominantly responsible for the binding observed.

We further showed that 10% of the dose of phenol added bound irreversibly upon incubation and that this binding, as in incubations with benzene, was prevented by GSH (Fig. 4). In incubations with benzene only 0.6% of the dose bound. Thus, although the dose of phenol was 40-fold higher than that of benzene, a 20-fold higher portion of the phenol dose bound. This is consistent with the interpretation of phenol being a metabolite more proximate to the binding species than benzene. Addition of UDPGA to incubation mixtures with [¹⁴C]benzene as substrate, decreased the amount of irreversibly bound metabolites as well as the amount of phenol (Fig. 6). An enzyme, UDP-glucuronyl transferase, is present in the microsomal fraction, which catalyzes the conjugation of UDPGA with acceptors of different sorts (24) including phenolic hydroxyl groups. Recently extensive conjugation was also found with benzo(a)pyrene-4,5-oxide and 7,8-oxide (25). The conjugation of the 4,5-oxide is thought to proceed via the diol and the conjugation with 7,8-oxide mainly by isomerization to 7-OH-benzpyrene before conjugation. Thus, to our present knowledge, the addition of UDPGA should have no effect on the irreversible binding if benzene oxide were the binding agent, while such an effect should be seen if a further metabolite of phenol bound. In Fig. 6 it appears as if UDPGA at low concentrations decreased the binding without significantly affecting the amount of phenol. A possible explanation for this is that at low UDPGA-concentrations the conjugation capacity is only enough to reduce the local concentration of phenol in the membranes, the critical compartment for the further enzymatic activation of phenol. At higher concentrations of UDPGA, the conjugation is so fast that an effect on the overall amount of phenol also is clearly seen.

On the basis of the findings described in this paper it can be concluded that benzene

oxide is metabolically formed from benzene, since addition of homogeneous epoxide hydratase led to easily quantifiable amounts of benzene dihydrodiol. The data are compatible with the concept that phenol formation occurs by isomerization of benzene oxide and not by direct insertion of oxygen. On the other hand the data show that benzene oxide is not trapped by glutathione under the conditions used (pH 7.5; absence of cytoplasmic fraction which contains glutathione S-transferases) and is not the metabolite responsible for the majority of the irreversible binding to macromolecules. What binds is instead an unknown product of the further oxidation of phenol. Whether or not these findings are of significance for the toxicity of benzene must be investigated in further studies, where the metabolism of benzene in the bone marrow and levels of different enzyme activities in this organ will be determined. If a metabolite of phenol is also the binding agent in bone marrow it could be that a deficient conjugating system in this organ is responsible for the peculiar organ specificity of benzene toxicity.

REFERENCES

1. Snyder, R. & Kocsis, J. J. (1975) *CRC Crit. Rev. Toxic.*, **3**, 265-288.
2. Gonasun, L. M., Witmer, C. M., Kocsis, J. J. & Snyder, R. (1973) *Toxicol. Appl. Pharmacol.*, **26**, 398-406.
3. Parke, D. V. & Williams, R. T. (1953) *Biochem. J.*, **54**, 231-238.
4. Porteous, J. W. & Williams, R. T. (1949) *Biochem. J.*, **44**, 56-60.
5. Parke, D. V. & Williams, R. T. (1953) *Biochem. J.*, **55**, 337-340.
6. Sato, R., Fukuyama, T., Suzuki, T. & Yoshikawa, J. (1963) *J. Biochem.*, **53**, 23-27.
7. Drew, R. T. & Fouts, J. R. (1974) *Toxicol. Appl. Pharmacol.*, **27**, 183-193.
8. Sloane, N. H. (1965) *Biochim. Biophys. Acta.*, **107**, 599-602.
9. Andrews, L. S., Lee, E. W., Witmer, C. M., Kocsis, J. J. & Snyder, R. (1977) *Biochem. Pharmacol.*, **26**, 293-300.
10. Oesch, F. (1973) *Xenobiotica*, **3**, 305-340.
11. Jerina, D. M. & Daly, J. W. (1974) *Science*, **185**, 573-582.
12. Sims, P. & Grover, P. L. (1974) *Adv. Cancer Res.*, **20**, 165-274.
13. Wiebel, F. J., Whitlock, J. P. & Gelboin, H. V. (1974) in *Survival in Toxic Environments*, (Khan, M. A. Q. & Bederka, J. P., eds.) pp. 261-293, Academic Press, New York.
14. Heidelberger, C. (1975) *Annu. Rev. Biochem.*, **44**, 79-127.
15. Jerina, D., Daly, J., Witkop, B., Zaltzman-Nirenberg, P. & Udenfriend, S. (1968) *Arch. Biochem. Biophys.*, **128**, 176-183.
16. Boyland, E. (1971) in *Handbook of Experimental Pharmacology, Concepts in Biochemical Pharmacology, Part 2* (Brodie, B. B. & Gillette, J. R., eds.), pp. 584-608, Springer-Verlag, Berlin.
17. Platt, K. & Oesch, F. (1977) *J. Label. Comp. Radiopharm.*, **13**, 471-479.
18. Platt, K. & Oesch, F. (1977) *Synthesis*, 449-450.
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265-275.
20. Bentley, P. & Oesch, F. (1975) *FEBS Lett.*, **59**, 291-295.
21. Jollow, D. J., Mitchell, J. R., Potter, W. Z., Davis, D. C., Gillette, J. R. & Brodie, B. B. (1973) *J. Pharmacol. Exp. Ther.*, **187**, 195-202.
22. Tomaszewski, J. E., Jerina, D. M. & Daly, J. W. (1975) *Biochemistry*, **14**, 2024-2031.
23. Daly, J. W., Jerina, D. M. & Witkop, B. (1972) *Experientia*, **28**, 1129-1264.
24. Dutton, G. J. (1971) in *Handbook of Experimental Pharmacology, Concepts in Biochemical Pharmacology, Part 2* (Brodie, B. B. & Gillette, J. R., eds.), pp. 378-400, Springer-Verlag, Berlin.
25. Nemoto, N. & Gelboin, H. V. (1976) *Biochem. Pharmacol.*, **25**, 1221-1226.
26. Oesch, F. (1974) *Biochem. J.*, **139**, 77-88.

MULTI-STEP METABOLIC ACTIVATION OF BENZENE.

Effect of superoxide dismutase on covalent binding to microsomal macromolecules, and identification of glutathione conjugates using high pressure liquid chromatography and field desorption mass spectrometry.

A. Tunek^a, K.L. Platt^b, M. Przybylski^c and F. Oesch^b.

a. Institute of Environmental Health, University of Lund,
Sölvegatan 21, S-223 62 Lund, Sweden.

b. Section on Biochemical Pharmacology, Institute of
Pharmacology, Obere-Zahlbacher-Strasse 67, D-6500 Mainz,
West Germany.

c. Institut für Organische Chemie der Universität,
Johann-Joachim-Becher-Weg 18-20, D-6500 Mainz, West Germany.

Running title: Mechanism of benzene toxicity.

SUMMARY

Incubation of ^{14}C -benzene or ^{14}C -phenol with liver microsomes from untreated rats, in the presence of a NADPH-generating system, gave rise to irreversible binding of metabolites to microsomal macromolecules. For both substrates this binding was inhibited by more than 50% by addition of superoxide dismutase to the incubation mixtures. The decrease in binding was compensated for by accumulation of ^{14}C -hydroquinone, indicating superoxide-mediated oxidation of hydroquinone as one step in the activation of benzene to metabolites binding to microsomal macromolecules. Since our previous work had shown that binding occurred mainly with protein rather than ribonucleic acid and was virtually completely prevented by glutathione, suggesting identity of metabolite(s) responsible for binding to protein and glutathione, a conjugate was chemically prepared from p-benzoquinone and reduced glutathione and identified by field desorption mass spectrometry as 2-(S-glutathionyl) hydroquinone. Microsomal incubations, containing an NADPH-generating system, with benzene, phenol, hydroquinone or p-benzoquinone in the presence of ^3H -glutathione or, alternatively, with ^{14}C -benzene or ^{14}C -phenol in the presence of unlabeled glutathione, were performed. All these incubations gave rise to a peak of radioactivity eluting from the high pressure liquid chromatograph at a retention time identical to that of the chemically prepared 2-(S-glutathionyl) hydroquinone, whilst microsomal incubation of catechol in the presence of ^3H -glutathione led to a conjugate with a very different retention time which was not observed after incubation of benzene or phenol. The microsomal metabolites of p-benzoquinone, hydroquinone and phenol thus eluting from the high pressure liquid chromatograph were further identified as the 2-(S-glutathionyl) hydroquinone by field desorption mass spectrometry. The mass spectra of the glutathione adduct formed from benzene during microsomal activation eluted from HPLC with the same retention time and also contained the molecular ion (MH^+) (m/e 416) of this conjugate as an intense peak, but the fragmentation patterns did not allow definitive assignments, probably due to the considerably smaller amounts of ultimate reactive metabolites formed from this pre-precursor and thus relatively large amounts of impurities.

The results indicate that rat liver microsomes activate benzene via phenol and hydroquinone to p-benzosemiquinone and/or p-benzoquinone as quantitatively important reactive metabolites.

INTRODUCTION

Benzene is a component of most motor fuels and therefore a widely spread environmental pollutant (1). The chronic toxicity of benzene is specifically directed towards the bone marrow and it is generally assumed that long-term exposure to benzene can induce leukemia (2, 3, 4).

The chronic toxicity of compounds that by themselves are chemically relatively inert is thought to originate from the metabolic formation of reactive intermediates, which are able to react with cellular macromolecules. The formation of such reactive intermediates has been extensively studied for several compounds, particularly aromatic polycyclic hydrocarbons (5-8). Although experimental data in the literature are somewhat conflicting, most scientists seem to agree that benzene also requires metabolic activation to exert its toxic effects (for reviews see (2) and (4)). It has also been reported that benzene metabolites will bind covalently to liver DNA in rats inhaling benzene (9), and that several organs of mice will contain irreversibly bound benzene metabolites after subcutaneous administration of benzene (10).

Recently, we reported irreversible binding of benzene metabolites to microsomal proteins after incubating rat liver microsomes with benzene (11). Although benzene oxide formation during the metabolism of benzene was demonstrated, it was found that the metabolite responsible for the main portion of the binding was not benzene oxide but a further metabolic product of newly formed phenol (11). Thus, the metabolic activation of benzene in rat liver microsomes is at least a two-step process.

Since it was found that GSH¹ prevented the irreversible binding, causing instead formation of water soluble products (11), it was assumed that the reactive intermediate, instead of binding to protein, formed a conjugate with GSH when this cofactor was present. In the present study HPLC¹ and FDMS¹ are used to compare the glutathione conjugates formed from benzene with those formed from phenol, catechol, hydroquinone and p-benzoquinone in

order to gain further insight into the pathway of the metabolic activation of benzene. In addition, the effects of superoxide dismutase on the covalent binding of ^{14}C -benzene and ^{14}C -phenol metabolites to macromolecules in microsomal incubations have been tested. Superoxide dismutase will decrease the concentration of superoxide anion in the incubation mixtures (12), and thereby inhibit the formation of radicals from e.g. aromatic dihydroxy compounds (13).

The results presented here show that semiquinones and/or quinones are quantitatively important reactive metabolites of benzene. Superoxide dismutase prevented the covalent binding of benzene metabolites by more than 50%, and data obtained with HPLC and FDMS strongly indicate that the GSH-conjugate formed from benzene in the microsomal incubations is 2-(S-glutathionyl) hydroquinone, i.e. a reaction product of GSH and p-benzosemiquinone or p-benzoquinone.

MATERIALS AND METHODS

Compounds

All chemicals, obtained from commercial sources, were of analytical grade, except for benzoquinone, which was synthetic grade, purified by steam distillation before use. L-glycine-1-³H-GSH was obtained from New England Nuclear and ¹⁴C-benzene and ¹⁴C-phenol from the Radiochemical Centre, Amersham. The specific activities were 2480, 100 and 35 mCi/mmol, respectively. Superoxide dismutase (from bovine blood) was obtained from Sigma, and had a specific activity of 2400 units/mg protein (12).

Preparation of microsomes

Liver microsomes from male Sprague-Dawley rats, weighing 200-250 g, were prepared as described (11). Protein concentrations were determined using the method of Lowry et al. (14) with bovine serum albumin as standard.

Incubations

The incubations contained: 1.5 ml of a buffer containing 100 mM Tris pH 7.5, 10 mM MgCl₂ and 10 μM MnCl₂; 2.4 mg microsomal protein, and when indicated a NADPH-generating system (3 mg glucose-6-phosphate; 2 mg NADP and 4 μg (0.56 U) glucose-6-phosphate dehydrogenase (Boehringer, Mannheim). The total volume was 3.0 ml.

¹⁴C-Benzene (21 nmol), ¹⁴C-phenol (11 nmol), benzene (4.5 μmol), phenol (0.3 μmol), hydroquinone (0.3 μmol), catechol (0.3 μmol) and p-benzoquinone (0.3 μmol) were added to the incubations dissolved in 10 μl acetone. ³H-GSH (0.9 μmol), GSH (9 μmol) and superoxide dismutase (amount indicated in each case) were added dissolved in 100 μl distilled water.

The incubation time was 30 min in the experiments when the effects of superoxide dismutase were tested, and 60 min when GSH-conjugates for HPLC- and FDMS-analysis were prepared.

Chemical reaction of p-benzoquinone with GSH

54 mg p-benzoquinone (0.5 mmol) was suspended in 3 ml distilled water. 154 mg GSH (0.5 mmol) was dissolved in 3 ml distilled water and added to the benzoquinone suspension. The mixture was stirred at room temperature for 2.5 h and thereafter frozen and lyophilized. FDMS was carried out directly on the lyophilized reaction mixture and on collected fractions after HPLC.

Isolation of metabolites when testing the effect of superoxide dismutase

In the experiments to test the effect of superoxide dismutase, metabolites were separated into water soluble, ethyl acetate-soluble and protein-bound compounds and counted by liquid scintillation spectrometry as previously described (11).

TLC¹ was performed on precoated aluminium sheets, silica gel 60 F₂₅₄, Merck. The sheets were developed with: A, benzene: methanol:acetic acid (70:8:4 v/v); B, benzene:dioxane:acetic acid (19:5:1 v/v) or C, benzene:chloroform:ethyl acetate (1:1:1 v/v). Table 1 shows R_f-values for phenol, hydroquinone, catechol and p-benzoquinone in the different thin layer systems. System A was routinely used to isolate and quantify phenol and hydroquinone as elsewhere described for phenol (11), and the results were occasionally controlled with systems B and C. A mixture of the four reference compounds was chromatographed together with each sample tested by TLC. As is evident from Fig. 3 below, ¹⁴C-hydroquinone could be isolated from incubations containing ¹⁴C-phenol, microsomes, a NADPH-generating system and superoxide dismutase. By making an aqueous solution of this ¹⁴C-hydroquinone and carrying out the extraction and isolation procedure, the recovery of hydroquinone was found to be 70%. With the same method we previously found the recovery for phenol to be 64% (11). All results given have been corrected for these recoveries.

HPLC-analysis

After incubation, all samples were extracted three times with 10 ml ethyl acetate in order to remove unmetabolized hydrocarbon and ethyl acetate-soluble metabolites that would complicate the HPLC elution profiles from incubations containing ^{14}C -benzene and ^{14}C -phenol. After extraction the water phases were frozen and lyophilized. The remainders after lyophilization were suspended in 1 ml of a water/ethanol mixture (3:2 v/v) and centrifuged at $2000 \times g$ for 10 min. 100 μl of the supernatant was injected into the HPLC.

The HPLC (Spectra-Physics) was equipped with a reversed phase (Spherisorb ODS, 10 μm) column (3 x 250 mm). The mobile phase consisted of a mixture of 2% acetic acid in water, and methanol. The flow rate was 0.4 ml/min. A linear solvent gradient, 0-95% methanol during 60 min, was applied. When radioactivity profiles were studied, the eluate was collected directly into scintillation vials, one minute per vial, for up to 120 min. The samples were then dissolved in 10 ml Unisolve (Koch-Light Laboratories, Colnbrooks Bucks, UK), and counted in a liquid scintillation counter. When samples were prepared for FDMS, the fraction eluting between 28-34 min after injection was collected. Prior to mass spectrometry these eluates were frozen at -90°C and lyophilized.

FDMS-analysis

FDMS was performed with a Varian MAT 711 double focusing spectrometer equipped with a combined e.i./f.i./f.d. ion source. Low resolution spectra were recorded with instrumental conditions as described previously (16). Activated 10 μm tungsten field ion emitters with an average needle length of 20-30 μm were prepared by the high temperature activation procedure (15). The lyophilized samples as DMSO solutions (approximately 1-2 $\mu\text{g}/\mu\text{l}$) were loaded on the emitter with a microliter syringe. The emitter was then introduced in the ion source by a direct insertion lock and heated gradually (at approximately 10 mA/min) from ambient to a maximum heating current of approximately 25 mA. Spectra

were recorded with an on-line Varian SS-100 data system on magnetic tape. Eight consecutive scans were averaged and evaluated for molecular and fragment ions. High resolution peak matching experiments were conducted at the BAT¹ for the molecular ion MH^+ of the benzoquinone-GSH conjugate determined in a preceding measurement with the same emitter. A resolution of approximately 7,000 was used, and tris-(perfluoroethyl)s-triazine (PCR, Gainesville, Fla., USA, m/e 435) as reference compound was admitted through a liquid reference inlet port.

RESULTS

Effect of superoxide dismutase on the covalent binding of metabolites of ^{14}C -benzene and ^{14}C -phenol to microsomal macromolecules

When ^{14}C -benzene was incubated with microsomes and a NADPH-generating system, addition of 2400 units superoxide dismutase decreased the covalent binding to macromolecules by 54% (Fig. 1). The decrease in binding was compensated for by accumulation of ^{14}C -hydroquinone. The formation of ^{14}C -phenol was unaffected by superoxide dismutase.

Fig. 2 shows similar results for ^{14}C -phenol. 2400 units superoxide dismutase decreased the binding by 64%, and this decrease was slightly overcompensated for by accumulation of ^{14}C -hydroquinone.

Incubations with ^{14}C -benzene or ^{14}C -phenol as substrate, with or without superoxide dismutase, were extracted with ethyl acetate and the extracts subjected to TLC. Resulting radioactivity profiles are shown in Fig. 3. As can be seen, hydroquinone and not catechol was accumulated in the presence of superoxide dismutase. The quantification of hydroquinone when the precursor benzene was substrate was rather uncertain due to the small amounts of metabolites, while the quantification when the immediate precursor phenol was substrate could be done much more accurately.

As 2400 units of superoxide dismutase were added to the incubations, their protein content was elevated by 42%. Therefore the possibility exists that the superoxide dismutase could trap some reactive metabolites simply by reaction of some functional groups of the protein with the reactive metabolite without enzyme catalysis. If then the superoxide dismutase were not completely precipitated by the ethanol treatment (see ref. 11), a decrease in binding to the precipitated material would be observed. However, two observations indicate that this is not the cause of the decrease in binding. First, the decreases in binding were fully compensated for the accumulation of ^{14}C -hydroquinone.

And second, if the superoxide dismutase was not totally precipitated, it would have remained in the water/ethanol phase and counted for radioactivity in the "water soluble fraction." The low amounts of radioactivity in the water/ethanol phase were, however, not affected by the addition of superoxide dismutase (not shown).

Chemical reaction of p-benzoquinone with GSH

When the GSH-solution was added to the suspension of the quinone, the yellow color of the quinone turned brownish. After only about 15 min, however, the reaction mixture was completely colorless, and white crystals remained after lyophilization.

When the lyophilized reaction mixture was analyzed directly with FDMS, the mono-GSH conjugate of hydroquinone could be definitely identified, while a bis-GSH conjugate could also be tentatively identified as a minor reaction product (details of the mass spectral studies are discussed below).

HPLC of an aliquot of the lyophilized reaction mixture gave a peak detectable by its absorption at 254 nm that eluted after 30-32 min. This fraction was collected, and the compound was identified by FDMS as the mono-GSH conjugate of hydroquinone (Fig. 5) (see below).

HPLC-analysis of GSH-conjugates

Benzene, phenol, catechol, hydroquinone and p-benzoquinone were incubated with rat liver microsomes in the presence of ^3H -GSH with or without a NADPH-generating system. In the same way ^{14}C -benzene and ^{14}C -phenol were incubated in the presence or absence of unlabeled GSH, with or without a NADPH-generating system. After incubation the samples were subjected to HPLC-analysis and fractions were collected and counted as described under "Materials and Methods."

Some results are shown in Fig. 4 a-f. In the presence of microsomes, GSH and a NADPH-generating system, ^{14}C -benzene and

^{14}C -phenol gave rise to a peak of radioactivity eluting with its maximum after 31-32 min (Fig. 4a and b). If GSH or the NADPH-generating system was omitted from the incubations, this peak was not found.

Unlabeled benzene, phenol, hydroquinone and p-benzoquinone also gave a peak with an identical retention time of 31-32 min when incubated with microsomes, ^3H -GSH and a NADPH-generating system (Fig. 4c-f). When no benzene or benzene derivatives were present, only the peak eluting after 3-6 min appeared. In the absence of a NADPH-generating system, benzene and phenol gave no trace of the peak eluting after 31-32 min. Hydroquinone, however, gave this peak also in the absence of this cofactor, although three-fold smaller than with NADPH. p-Benzoquinone formed the peak eluting after 31-32 min apparently independent of NADPH-generating system.

Catechol, when incubated as described above with ^3H -GSH, gave a different elution profile, with a very small peak eluting after 50-54 min.

Identification of conjugation products by FDMS

In order to identify the possible conjugation products formed in the microsomal oxidation of benzene and its metabolites in the presence of GSH, the mass spectra of model compounds obtained by reacting p-benzoquinone with GSH at a 1:1 molar ratio were investigated and compared with those obtained from the microsomal incubations. The mass spectrum of the lyophilized reaction mixture of benzoquinone and GSH was first studied and was compared with the conjugate isolated by HPLC from this reaction mixture (Fig. 5). Both spectra were dominated by ions at m/e 416 corresponding to the protonated molecular ion MH^+ of 2-(S-glutathionyl)hydroquinone. High resolution peak-matching studies of these ions revealed the correct formula, $\text{C}_{16}\text{H}_{22}\text{N}_3\text{O}_8\text{S}$ (416.1156, $\Delta = 2.8$ mmu). Fragment ions observed at 14 mA emitter heating current, which was slightly above the BAT^1 of m/e 416 were identical in both spectra and were fully consistent with previous FDMS

studies of GSH conjugates of electrophilic reactive drugs and their metabolites (16, 17). Thus, a typical cleavage at the γ -Glu-Cys' peptide bond leads to ions at m/e 286 (protonated Cys-S(hydroquinone)-Gly) and m/e 130 (γ -Glu). The fragment at m/e 398 can be assigned to the loss of water, while a rather prominent ion at m/e 361, which was found in the spectra of all investigated conjugates, is either an unknown fragment or an impurity eluting from the HPLC with the same retention time. The minor ions at m/e 308 and 110 may be due to protonated glutathione and hydroquinone. In addition to the identification of the mono-GSH conjugate of hydroquinone purified by HPLC, the mass spectrum of the reaction mixture of GSH and p-benzoquinone showed an ion at m/e 721 which would be consistent with MH^+ of a hydroquinone-bis(GSH) conjugate. However, attempts to obtain precise mass measurements of this ion by the peak matching technique at various emitter heating currents failed because of its instability and rapid decomposition.

Benzene, phenol, hydroquinone and p-benzoquinone were incubated with rat liver microsomes, 3 mM GSH and a NADPH-generating system. Fractions eluting from the HPLC-column with an elution time of 28-34 min were collected and prepared for FDMS as described under "Materials and Methods."

The mass spectral investigation of the microsomal metabolite conjugates from p-benzoquinone and hydroquinone isolated by HPLC (Fig. 6a, b) revealed the same intense ions at m/e 416 as the MH^+ ion for the authentic reaction product of benzoquinone and GSH (Fig. 5). Moreover, the fragments at m/e 286 and 130, typical for peptide cleavage of the γ -Glu-Cys bond, were also present (Fig. 6a, b). The fragment at m/e 84 can be assigned to loss of CO_2 from the fragment at m/e 130 and was also present in the spectrum of the chemically prepared standard (although not shown in Fig. 5). The fragment at m/e 398 corresponding to loss of water from the MH^+ was also present. The ion at m/e 78 corresponds to the solvent dimethylsulfoxide. Since metabolism of pre-precursors (phenol, benzene) leads to a lower amount of

ultimate, reactive metabolite(s) compared with the incubation of the immediate precursor, the ratio of the concentration of the conjugate to that of impurities becomes more unfavourable leading to overshadowing of the typical fragmentation patterns in the mass spectra by still other fragments. The mass spectrum of a corresponding metabolite obtained from phenol at an identical retention time on HPLC also showed an abundant ion at m/e 416 (MH^+) and prominent peaks at m/e 130 (pyroglutamyl ion), and 286 (protonated Cys-S-(hydroquinone)-Gly) (Fig. 6c). A small amount of a microsomal metabolite derived from the, even more distant pre-precursor benzene, which eluted with an identical retention time as the hydroquinone and benzoquinone conjugates on HPLC, was also investigated by FDMS. This mass spectrum did also contain an intense peak at m/e 416 (MH^+) but no clear fragmentation pattern could be established.

DISCUSSION

The pathways for metabolic activation of many aromatic hydrocarbons have recently been found to be more complicated than was earlier thought. Secondary or even tertiary metabolites such as diol-epoxides, phenol-epoxides, quinones or semiquinones have been found to contribute more than the primary arene oxides to the observed covalent binding to macromolecules. Such evidence has been presented for, e.g., benzo(a)pyrene (18, 19), polychlorinated biphenyls (20), naphthalene (21), 3-methylcholanthrene (22) and benzene (11).

Since we have found phenol to be an intermediate in the metabolic activation of benzene (11), the fate of phenol in the organism is of interest for the further clarification of this process. When administered in vivo, phenol is to a large extent directly conjugated and excreted. Depending on species and amount given, however, up to 7% of the phenol dose will be further oxygenated to form hydroquinone (23, 24). In an early study catechol also was reported to be formed in small amounts (25). Thus, hydroquinone is the main further oxygenated metabolite of phenol, while catechol might be formed in trace amounts.

Several aromatic dihydroxy compounds have been shown to possess the potential of forming semiquinones and quinones. The microsomal metabolic activation of α -methyldopa (26), 2-hydroxysterone (27, 28) and ethynyl estradiol (29) has been investigated, and the main reactive metabolites are thought to be semiquinones or quinones. The semiquinones are probably generated from the dihydroxy compounds via the mediation of superoxide radicals (13, 26) and the quinones after further oxidation of the semiquinones by some electron acceptor (30). On the other hand, quinones can form semiquinones by reductive mechanisms dependent on NADPH or NADH (31), or dihydroxy compounds in a two-electron transfer process via the mediation of DT-diaphorase (32). Furthermore, semiquinones can undergo dismutation, i.e., two semiquinone molecules form one quinone and one hydroquinone

molecule (30, 33).

The only function known for the enzyme superoxide dismutase is to lower the concentration of superoxide anion by causing its dismutation (12). Thus, since the formation of semiquinones from dihydroxy compounds is thought to be dependent on superoxide anion (13, 26), superoxide dismutase should block the radical formation from, e.g., hydroquinone.

In this paper it is shown that hydroquinone could be isolated as a metabolite of benzene and phenol, but in appreciable amounts only in the presence of superoxide dismutase. In the absence of this enzyme almost all the hydroquinone was apparently transformed to compounds covalently binding to macromolecules. This indicates that hydroquinone is transformed to p-benzosemiquinone and/or p-benzoquinone by superoxide anions in microsomal incubations containing NADPH, and that the semiquinone and/or the quinone almost completely bind to macromolecules. Furthermore, since the covalent binding obtained in incubations with benzene or phenol was inhibited by more than 50% by superoxide dismutase, it can be concluded that p-benzosemiquinone and/or p-benzoquinone are quantitatively important reactive metabolites of benzene and phenol.

Electrophilic reactive metabolites can be inactivated by conjugation with GSH. This inactivation is mediated by GSH transferases (34), primarily located in the cytosolic fraction but recently shown to be present also in microsomes (35, 36, 37). However, cysteine is often just as effective as GSH in preventing binding of electrophilic reactive metabolites to macromolecules. This indicates that many reactive metabolites will react spontaneously with sulfhydryl-containing agents, since cysteine is not accepted as a substrate by GSH transferases. We showed that cysteine could effectively prevent covalent binding of benzene metabolites to macromolecules in microsomal incubations (11). It is therefore likely that the GSH conjugates formed during microsomal metabolism of benzene result from spontaneous chemical reactions.

In the second part of this study we have attempted to trap the reactive metabolites of benzene by GSH, in order to obtain defined conjugates for HPLC- and FDMS-studies.

Since the findings described above indicated that p-benzo-semiquinone and/or p-benzoquinone were reactive metabolites of benzene and phenol, we tried to synthesize a GSH-conjugate by reacting p-benzoquinone with GSH. p-Benzoquinone has been shown to react spontaneously with thioglycolic acid, in a multi-step reaction, to form eventually hydroquinone tetrathioglycolic acid when the two compounds reacted at a 1:1 molar ratio (38). When we mixed p-benzoquinone and GSH under similar conditions, the mono-GSH hydroquinone conjugate could be definitely identified (Fig. 5) and a minor bis-GSH conjugate tentatively identified by FDMS. The formation of bis(GSH) conjugates as well as bis(cysteinylyl) conjugates has been suggested from previous studies on reactive acridine derivatives (40). When benzene, phenol, hydroquinone or p-benzoquinone were incubated with ^3H -GSH, microsomes and a NADPH-generating system, or ^{14}C -benzene or ^{14}C -phenol with unlabeled GSH under the same conditions, HPLC elution profiles revealed that with benzene and all these derivatives a compound had been formed which eluted at an identical retention time (Fig. 4a-f). The retention time was the same as for the synthetic mono-GSH hydroquinone conjugate described above, but very different from the retention time of the GSH-conjugate of a metabolically activated related compound, catechol. p-Benzoquinone formed this compound independently of a NADPH-generating system, while hydroquinone also formed small amounts without NADPH, possibly due to autoxidation of the hydroquinone. For the formation of the reactive metabolite from benzene and phenol, NADPH was an absolute requirement. The GSH-conjugates of the microsomal metabolites from p-benzoquinone and hydroquinone could be definitely identified by FDMS to be the mono-GSH hydroquinone conjugate (Fig. 6a, b). This could arise either by direct reaction of GSH and p-benzoquinone, or by reaction of GSH and p-benzosemiquinone followed by oxidation. The mass spectrum of the GSH-conjugate of the phenol metabolite also contained an abundant ion at m/e 416 (MH^+ of the mono-GSH

hydroquinone conjugate) and prominent fragment peaks at m/e 130 (pyroglutamyl ion) and 286 (protonated Cys-S-(hydroquinone)-Gly), but otherwise the fragmentation pattern was less clear than that of the first two conjugates. The mass spectrum of the GSH conjugate of the benzene metabolite did also contain an intensive peak at m/e 416 but the fragmentation pattern was not favourable for definitive assignments.

The fact that the mass spectra of the GSH-conjugate formed from metabolically activated phenol and, particularly, benzene are more complicated is not surprising, since more metabolic steps are required and thus the quantities of the conjugate will be smaller with relatively larger amounts of impurities. Nevertheless, the combination of the results of the HPLC- (same retention time under conditions where a closely related adduct had a very different retention time) and FDMS-studies (MH^+ in all spectra, key fragments prominent in the spectra of the conjugate of metabolically activated phenol, hydroquinone, p-benzoquinone and chemically prepared standard) and our previous study (11), which indicated that a metabolite of phenol is mainly responsible for the binding of metabolically activated benzene to protein, strongly indicates that the same GSH- conjugate was formed from metabolically activated benzene, phenol and hydroquinone as is formed from benzoquinone in presence or absence of a metabolically activating system. p-Benzosemiquinone and p-benzoquinone would form identical GSH conjugates, and therefore it is not possible to decide whether both or which of the two compounds reacted with GSH in the microsomal incubations.

The results of this study strongly indicate that p-benzosemiquinone and/or p-benzoquinone are reactive metabolites of benzene in a microsomal incubation system. It is therefore interesting to note a recent report showing the production of superoxide radicals in rabbit liver to be 3.5-fold the production in the bone marrow, while the superoxide dismutase activity was 12-fold higher in the liver (39). This might have the consequence

that bone marrow is more susceptible to toxicity of compounds with the ability to form radicals like semiquinones.

Fig. 7 shows the scheme for the metabolic activation of benzene suggested by the results of this study. All intermediates in the scheme could in vivo be subjected to conjugation reactions. It is therefore not possible, on the basis of available data, to assess whether or not the suggested metabolic activation pathway is of importance for the development of the bone marrow toxicity of benzene. More in vivo experiments with animals exposed to benzene, and with different modulators influencing the metabolism of this hydrocarbon are needed to elucidate the molecular mechanism for benzene toxicity.

ACKNOWLEDGEMENT

We wish to thank Thomas Friedberg, Section on Biochemical Pharmacology, Mainz, Germany, for several helpful discussions. This work was supported by the Swedish Cancer Society and the Stiftung Volkswagenwerk.

REFERENCES

1. M. Berlin, J.C. Gage and E. Johnson. Increased aromatics in motor fuels: A review of the environmental and health effects. *Work-environm.-hlth.* 11 (1974) 1.
2. R. Snyder and J.J. Kocsis. Current concepts of benzene toxicity. *CRC Crit. Rev. Toxic.* 3 (1975) 265.
3. R. Snyder, E.W. Lee, J.J. Kocsis and C.M. Witmer. Bone marrow depressant and leukemogenic actions of benzene. *Life Sciences* 21 (1977) 1709.
4. S. Laskin and B.D. Goldstein. Benzene toxicity, a critical evaluation. *J. Tox. Env. Health*, suppl. 2 (1977).
5. F. Oesch. Mammalian epoxide hydrases: Inducible enzymes catalyzing the inactivation of carcinogenic and cytotoxic metabolites derived from aromatic and olefinic compounds. *Xenobiotica* 3 (1973) 305.
6. D. Jerina and J.W. Daly. Arene oxides: A new aspect of drug metabolism. *Science* 185 (1974) 573.
7. P. Sims and P.L. Grover. Epoxides in polycyclic aromatic hydrocarbon metabolism and carcinogenesis. *Adv. Cancer Res.* 20 (1974) 165.
8. C. Heidelberger. Chemical Carcinogenesis. *Ann. Rev. Biochem.* (1975) 79.
9. W.K. Lutz and C. Schlatter. Mechanism of the carcinogenic action of benzene: Irreversible binding to rat liver DNA. *Chem.-Biol. Interact.* 18 (1977) 241.

10. R. Snyder, E.W. Lee and J.J. Kocsis. Binding of labeled benzene metabolites to mouse liver and bone marrow. *Res. Com. Chem. Path.* 20 (1978) 191.
11. A. Tunek, K.L. Platt, P. Bentley and F. Oesch. Microsomal metabolism of benzene to species irreversibly binding to microsomal protein and effects of modifications of this metabolism. *Mol. Pharmacol.* 14 (1978) 920.
12. J.M. McCord and I. Fridovich. Superoxide dismutase: An enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* 244 (1969) 6049.
13. C.C. Winterbourn, J.K. French and R.F.C. Claridge. Superoxide dismutase as an inhibitor of reactions of semiquinone radicals. *FEBS Lett.* 94 (1978) 269.
14. O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193 (1951) 265.
15. H.O. Beckey and H.R. Schulten. Field desorption mass spectrometry. *Angew. Chem.* 87 (1975) 425.
16. M. Przybylski, I. Lüderwald, E. Kraas, W. Voelter and S.D. Nelson. Field desorption mass spectra of gastrine peptides and glutathione derivatives. *Z. Naturforsch.* 346 (1979) 736.
17. M. Przybylski, R.H. Adamson, J.A.R. Mead and R.L. Csyk. Field desorption mass spectrometric identification of the conjugation products of m-AMSA (NSC-141549) with endogenous thiols in vitro and in rat bile. *Proc. Am. Assoc. Cancer Res.* 20 (1979) 1165.

18. O. Pelkonen, A.R. Boobis, H. Yagi, D.M. Jerina and D.W. Nebert. Tentative identification of benzo(a)pyrene metabolite-nucleoside complexes produced in vitro by mouse liver microsomes. *Mol. Pharmacol.* 14 (1978) 306.
19. B. Jernström, S. Orrenius, O. Undeman, A. Gräslund and A. Ehrenberg. Fluorescence study of DNA-binding metabolites of benzo(a)pyrene formed in hepatocytes isolated from 3-methylcholanthrene-treated rats. *Cancer Res.* 38 (1978) 2600.
20. S. Hesse, M. Mezger and T. Wolff. Activation of ^{14}C -chlorobiphenyls to protein-binding metabolites by rat liver microsomes. *Chem.-Biol. Interact.* 20 (1978) 355.
21. S. Hesse and M. Mezger. Involvement of phenolic metabolites in the irreversible protein-binding of aromatic hydrocarbons: Reactive metabolites of ^{14}C -naphthalene and ^{14}C -naphthol formed by rat liver microsomes. *Mol. Pharmacol.* 16 (1979) 667.
22. A. Eastman and E. Bresnick. Metabolism and DNA binding of 3-methylcholanthrene. *Cancer Res.* 39 (1979) 4316.
23. I.D. Capel, M.R. French, P. Millburn, R.L. Smith and R.T. Williams. The fate of ^{14}C -phenol in various species. *Xenobiotica* 2 (1972) 25.
24. J. Kao and J.W. Bridges. Metabolism of ^{14}C -phenol by sheep, pig and rat. *Xenobiotica* 9 (1979) 141.
25. D.V. Parke and R.T. Williams. Studies in detoxication. 54. The metabolism of benzene. a. The formation of phenylglucuronide and phenylsulphuric acid from ^{14}C -benzene. b. The metabolism of ^{14}C -phenol. *Biochem. J.* 55 (1953) 337.

26. E. Dybing, S.D. Nelson, J.R. Mitchell, H.A. Sasame and J.R. Gillette. Oxidation of α -methyldopa and other catechols by cytochrome P-450-generated superoxide anion: possible mechanism of methyldopa hepatitis. *Mol. Pharmacol.* 12 (1976) 911.
27. F. Marks and E. Hecker. Metabolism and mechanism of action of oestrogens. XII. Structure and mechanism of formation of water-soluble and protein-bound metabolites of oestrone in rat liver microsomes in vitro and in vivo. *Biochim. Biophys. Acta* 187 (1969) 250.
28. F. Marks and E. Hecker. Stoffwechsel von 4-¹⁴C-2-Hydroxy-östron in Rattenlebermikrosomen. *Hoppe-Seyler's Z. Physiol. Chem.* 350 (1979) 69.
29. H.M. Bolt and H. Kappus. Irreversible binding of ethynyl-estradiol metabolites to protein and nucleic acids as catalyzed by rat liver microsomes and mushroom tyrosinase. *J. Steroid Biochem.* 5 (1974) 179.
30. T. Ohnishi, H. Yamazaki, T. Iyanagi, T. Nakamura and I. Yamazaki. One-electron-transfer reactions in biochemical systems. II. The reaction of free radicals formed in the enzymic oxidation. *Biochim. Biophys. Acta* 172 (1969) 357.
31. N.R. Bachur, S.L. Gordon and M.V. Gee. A general mechanism for microsomal activation of quinone anticancer agents to free radicals. *Cancer Res.* 38 (1978) 1745.
32. L. Ernster, L. Danielsson and M. Ljunggren. DT Diaphorase. I. Purification from the soluble fraction of rat-liver cytoplasm, and properties. *Biochim. Biophys. Acta* 58 (1962) 171.

33. I. Yamazaki and L.H. Piette. Mechanism of free radical formation and disappearance during the ascorbic acid oxidase and peroxidase reactions. *Biochim. Biophys. Acta* 50 (1961) 62.
34. I. Arias and W.B. Jacoby. Glutathione: Metabolism and function. Kroe Foundation Series, vol. 6, Raven Press, New York, 1976.
35. H.R. Glatt and F. Oesch. Inactivation of electrophilic metabolites by glutathione S-transferases and limitation of the system due to subcellular localization. *Arch. Toxicol* 39 (1977) 87.
36. T. Friedberg, P. Bentley, P. Stasiecki, H.R. Glatt, D. Raphael and F. Oesch. The identification, solubilization and characterization of microsome-associated glutathione S-transferases. *J. Biol. Chem.* 254 (1979) 12028.
37. R. Morgenstern, J.W. DePierre and L. Ernster. Activation of microsomal glutathione S-transferase activity by sulphydryl reagents. *Biochem. Biophys. Res. Commun.* 87 (1979) 657.
38. M. Schubert. The interaction of thiols and quinones. *J. Am. Chem. Soc.* 69 (1947) 712.
39. S.K. Hanson, M.W. Anders and M. R. Montgomery. Oxygen metabolism in rabbit bone marrow and liver. *Biochem. Biophys. Res. Commun.* 85 (1978) 783.
40. F. Wild and J.M. Young. The reaction of mepacrine with thiols. *J. Chem. Soc. B* (1965) 7261.

FOOTNOTES

1. Abbreviations used: GSH, reduced glutathione; HPLC, high performance liquid chromatography; FDMS, field desorption mass spectrometry; TLC, thin layer chromatography; BAT, best anode temperature (temperature of the field ion emitter at which the highest intensity of the molecular ion is observed).

Table 1. R_f -values in the thin layer solvent systems*.

Compound	R_f -values		
	Solvent system**		
	A	B	C
Phenol	0.41	0.51	0.54
Hydroquinone	0.15	0.24	0.33
Catechol	0.23	0.31	0.38
p-Benzoquinone	0.61	0.58	0.54

*The compounds were applied dissolved in ethyl acetate and detected in UV-light. The sheets were developed 15 cm.

**See "Materials and Methods."

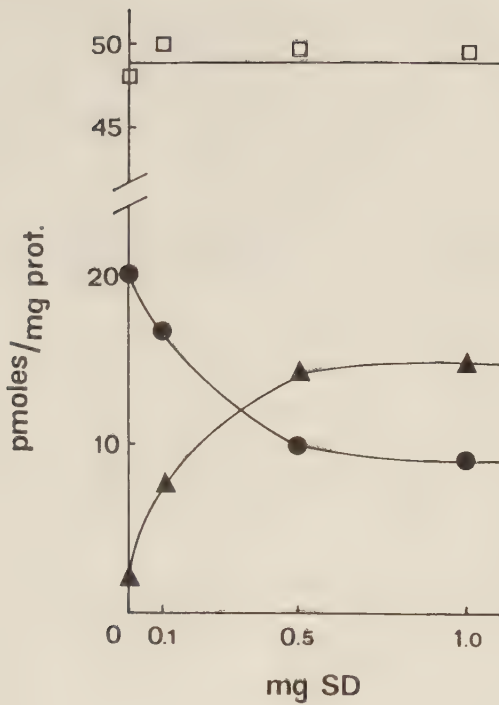


Fig. 1. Effect of superoxide dismutase (SD) on phenol formation (□), hydroquinone formation (▲) and irreversible binding (●) with 21 nmol 14 C-benzene as substrate.

Incubations were performed in the presence of a NADPH-generating system as described under "Materials and Methods". After incubating 30 min without superoxide dismutase and a NADPH-generating system the radioactivity of the different fractions corresponded to the following amounts of metabolites (given as pmol/mg microsomal protein): phenol 8.5, hydroquinone 1.0 and irreversibly bound 3.5. The results given in the figure represent the difference between measured values and values obtained without a NADPH-generating system. The determinations were made in duplicate. For phenol formation and irreversible binding the duplicate experiments differed at the most 8% from each other, while the variation when measuring hydroquinone was up to 20%.

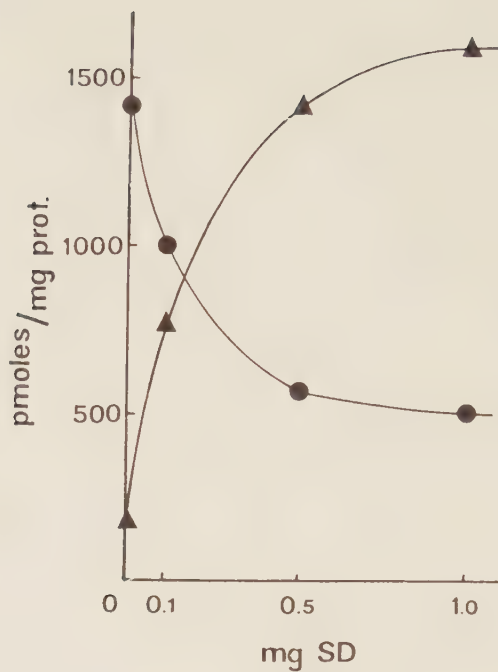


Fig. 2. Effect of superoxide dismutase (SD) on hydroquinone formation (▲) and irreversible binding (●) with 11 nmol ^{14}C -phenol as substrate.

See also legend to Fig. 1. In the absence of superoxide dismutase and a NADPH-generating system the following values were obtained (pmol/mg microsomal protein): hydroquinone 9 and irreversible binding 110. Duplicate experiments differed by less than 8%.

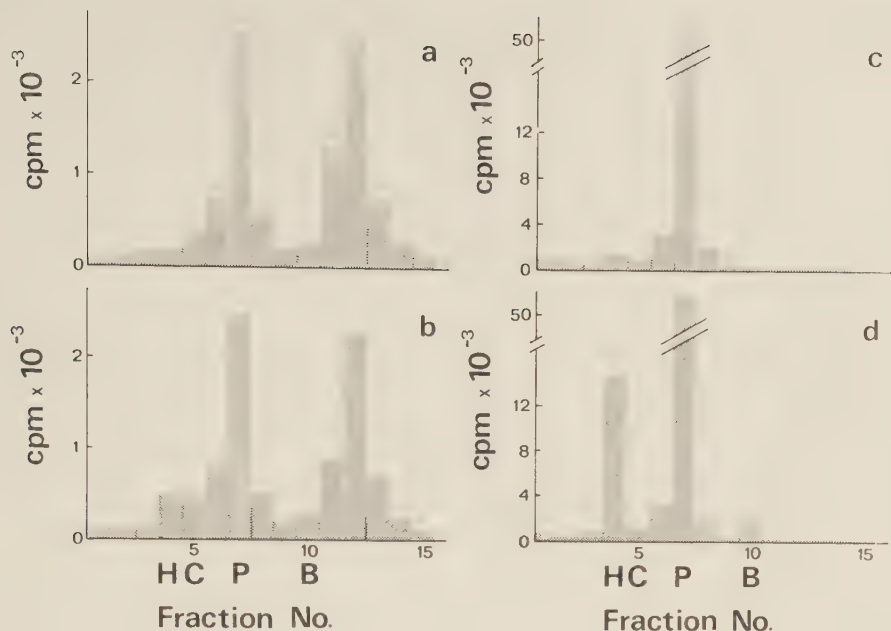


Fig. 3. Radioactivity profiles after TLC of ethyl acetate extracts from incubations containing microsomes, a NADPH-generating system and a. 21 nmol ^{14}C -benzene, b. 21 nmol ^{14}C -benzene and 2400 units superoxide dismutase, c. 11 nmol ^{14}C -phenol and d. 11 nmol ^{14}C -phenol and 2400 units superoxide dismutase.

For further details on the incubations see "Materials and Methods". Solvent system A was used, and radioactivity counted as earlier described (11). Indicated are migration distances of the reference compounds hydroquinone (H), catechol (C), phenol (P) and p-benzoquinone (B). A mixture of these reference compounds was placed on the same spot on the thin layer plate as the samples. The radioactivity in fractions 10-14 in a. and b. represents unmetabolized ^{14}C -benzene.

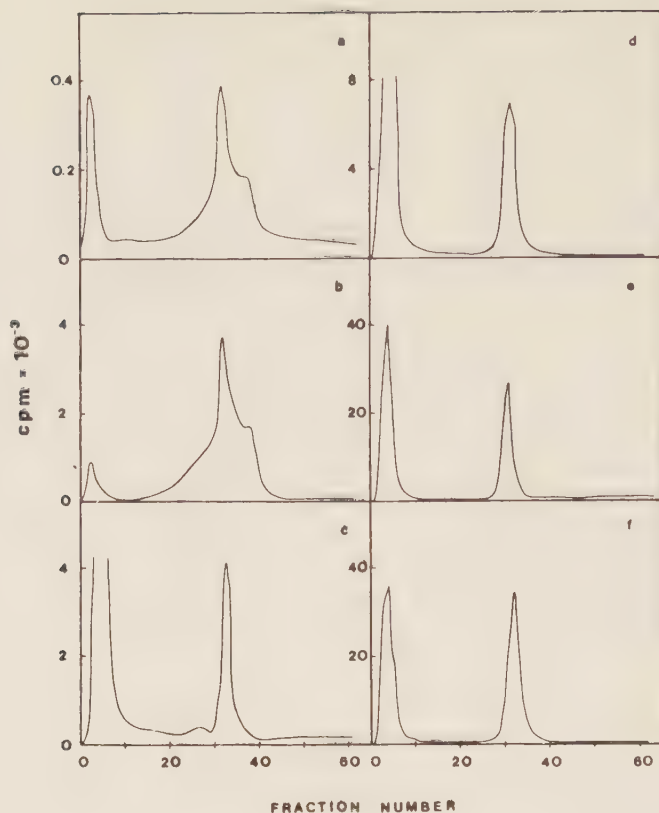


Fig. 4. HPLC elution profiles.

The incubations contained: a. 21 nmol ^{14}C -benzene and 9 μmol GSH, b. 11 nmol ^{14}C -phenol and 9 μmol GSH, c. 4.5 μmol benzene and 0.9 μmol ^3H -GSH, d. 0.3 μmol phenol and 0.9 μmol ^3H -GSH, e. 0.3 μmol hydroquinone and 0.9 μmol ^3H -GSH and f. 0.3 μmol p-benzoquinone and 0.9 μmol ^3H -GSH. Incubations with a NADPH-generating system, HPLC-analysis and counting were carried out as described under "Materials and Methods".

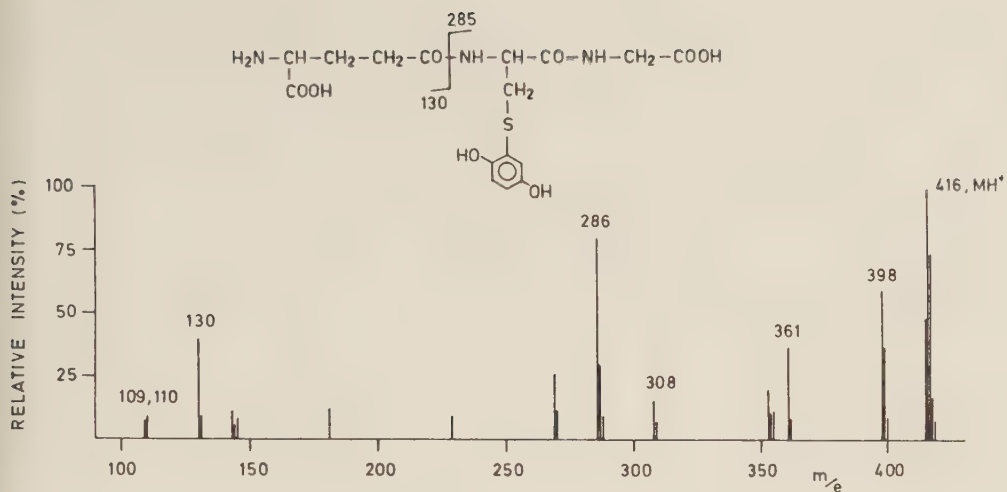


Fig. 5. Field desorption mass spectrum of the conjugate isolated by HPLC from the reaction mixture of p-benzoquinone and GSH. Details on reaction conditions, HPLC and FDMS are given under "Materials and Methods".

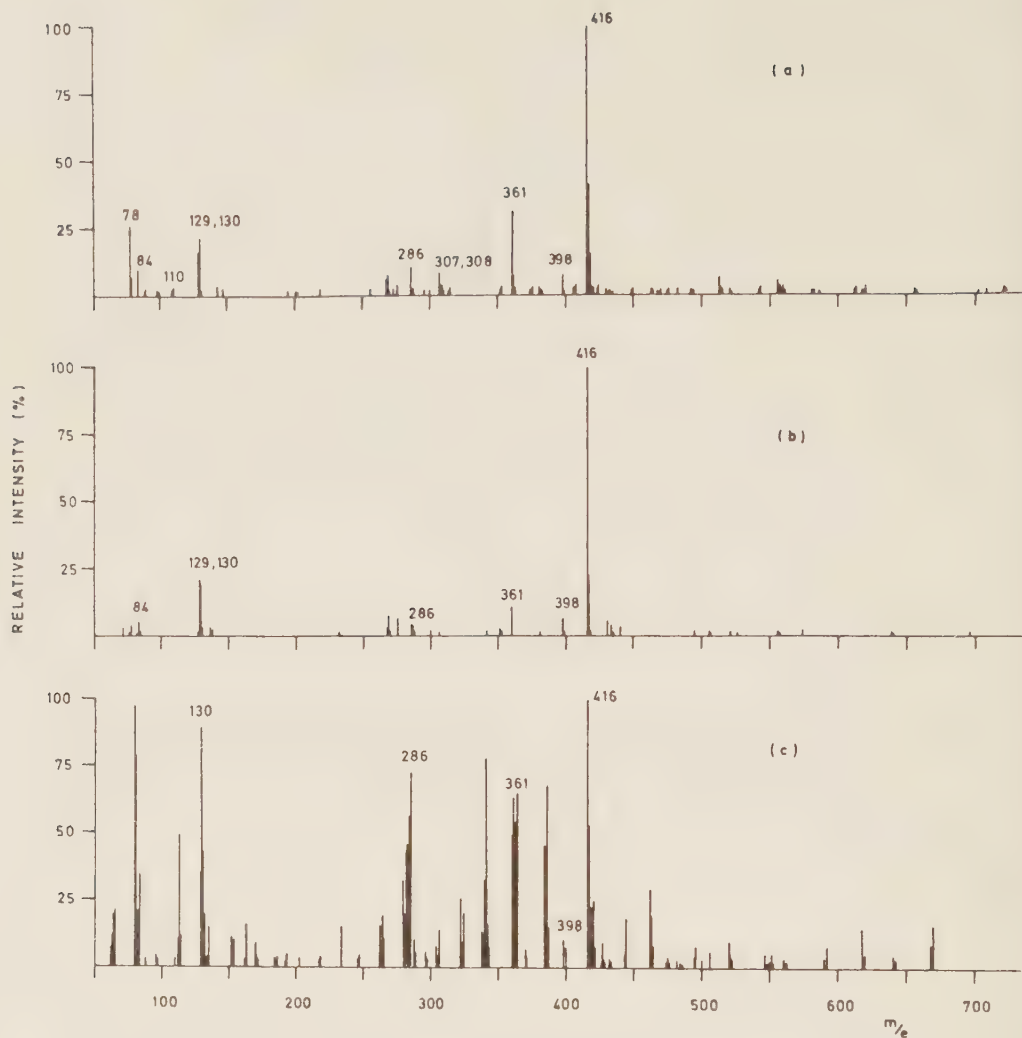


Fig. 6. Field desorption mass spectra of a. the GSH-conjugate of the microsomal metabolite of p-benzoquinone, 13 mA emitter heating current, b. the GSH-conjugate of the microsomal metabolite of hydroquinone, 14 mA emitter heating current, c. the GSH-conjugate of the microsomal metabolite of phenol, 14 mA emitter heating current. Incubations, HPLC and FDMS were performed as described under "Materials and Methods". 9 μ mol GSH, microsomes and a NADPH-generating system were present in the incubations.

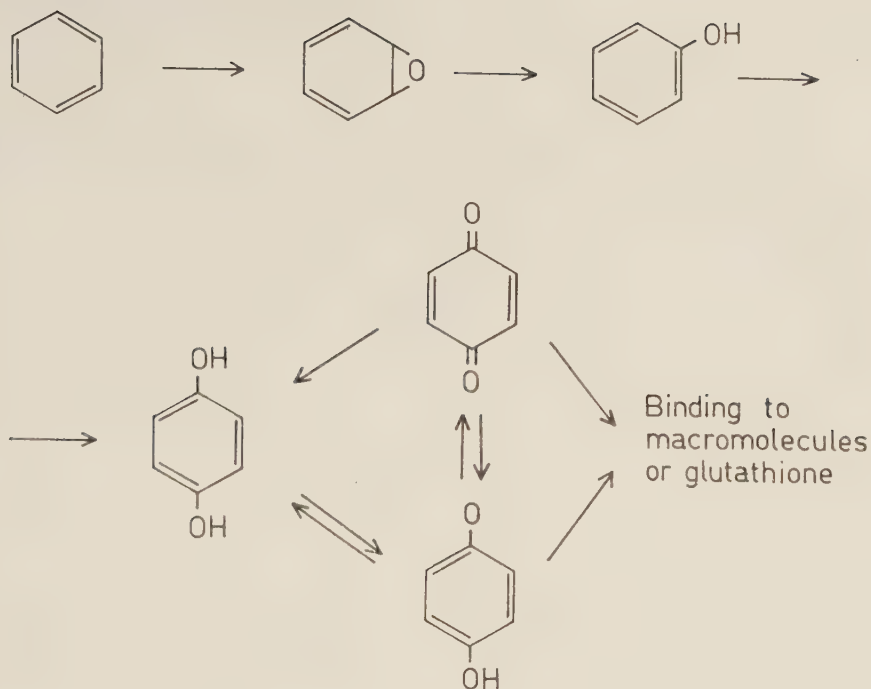


Fig. 7. Proposed scheme for the metabolic activation of benzene by rat liver microsomes.

MICROSOMAL TARGET PROTEINS OF METABOLICALLY ACTIVATED AROMATIC HYDROCARBONS

A. TUNEK, C. SCHELIN^a and B. JERGIL^{a,*}

Institute of Environmental Health, University of Lund and ^aBiochemistry 1, Chemical Centre, University of Lund, S-220 07 Lund (Sweden)

(Received 28th March, 1979)

(Accepted 4th June, 1979)

SUMMARY

The specificity of binding to microsomal proteins of metabolically activated hydrocarbons has been studied. Radioactively labelled benzene, phenol, chlorobenzene, BP and MC were incubated with liver microsomes from control, phenobarbital- and MC-treated rats in the presence of an NADPH-generating system. The patterns of metabolite binding to microsomal proteins were examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and fluorography. Benzene, phenol and chlorobenzene metabolites showed one type of binding pattern dominated by a band at 72 000 M_r . This band was strong both in control and induced microsomes. Additional radioactive bands were seen in the 50 000–60 000 M_r region particularly in MC-induced microsomes. BP and MC metabolites showed a different type of binding pattern with incorporation of radioactivity into several fractions in the 50 000–60 000 M_r region of MC-induced microsomes. Two other strongly labelled fractions occurred at 68 000 and 72 000 M_r . The incorporation was low into control and phenobarbital-induced microsomes. Two labelled bands (M_r 56 000 and 72 000) were common for all hydrocarbons in MC-induced microsomes. The 56 000 M_r band had the same mobility in the gel as an MC-induced protein likely to be cytochrome *P*-448. The NADPH-generating system was essential for metabolite binding and GSH and UDPGA greatly reduced binding. We suggest that differences in metabolite binding patterns reflect differences in the routes of metabolite formation and that activated hydrocarbons are likely to bind to proteins close to their site of formation.

* To whom correspondence should be sent.

Abbreviations: BP, benzo[*a*]pyrene; GSH, reduced glutathione; MC, 3-methylcholanthrene; SDS, sodium dodecylsulfate; UDPGA, UDP-glucuronic acid.

INTRODUCTION

Many toxic and/or carcinogenic compounds exert their adverse effects only after metabolic activation [1]. The reactive metabolites thus formed can bind covalently to cellular macromolecules and disturb normal cellular functions, giving rise to cytotoxic effects, mutation or cancer [2,3]. Most such compounds are metabolised by the mixed-function oxidase system located principally in the endoplasmic reticulum.

There has been a particularly intense interest in the covalent binding of activated hydrocarbons to DNA, since it seems reasonable to assume that carcinogenic and mutagenic effects originate from such interactions. However, covalent binding to microsomal macromolecules by metabolites of a large number of compounds has also been demonstrated after incubation with microsomes [4]. Metabolites of some compounds, e.g. 4-chlorobiphenyl, bind preferentially to ribosomal RNA [5], and others, such as benzene, bind almost exclusively to proteins [6]. Massive covalent binding to macromolecules is thought to contribute to cytotoxicity, as suggested for e.g. bromobenzene [3].

While there has been a great interest in the binding of metabolites to a variety of macromolecules, there have been no reports on the specificity of binding to proteins at the site of activation, i.e. to microsomal proteins. In this study we have incubated radioactively labelled hydrocarbons with rat liver microsomes and examined the specificity of metabolite binding to microsomal proteins by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

MATERIALS AND METHODS

All chemicals used were of analytical grade. [^{14}C]Benzene, [^{14}C]phenol, [^{14}C]BP and [^3H]MC were from the Radiochemical Centre, Amersham.

Male Sprague-Dawley rats, weighing approx. 150 g, were obtained from Anticimex, Stockholm. They were maintained on a standard chow diet and free access to water. Phenobarbital, 80 mg/kg, was injected i.p. on three consecutive days and the animals were sacrificed on the fourth day. MC, 80 mg/kg in corn oil, was injected i.p. 24 h before sacrifice. Liver microsomes were prepared as described earlier [6]. Protein was determined by the Lowry procedure [7] using bovine serum albumin as standard.

The incubation mixture used for studies of hydrocarbon binding to microsomal macromolecules contained 0.6 ml of buffer (100 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 and 10 μM MnCl_2), an NADPH-generating system (2 mg glucose 6-phosphate, 1.5 mg NADP and 2.7 μg (37 U) glucose-6-phosphate dehydrogenase), radiolabelled hydrocarbons and 1.2–1.8 mg microsomal protein in a total volume of 1.2 ml. The hydrocarbons were added dissolved in 10 μl acetone in amounts and specific activities as indicated in each case. The incubations were carried out at 37°C for 60 min, unless otherwise is stated. When used, GSH or UDPGA were administered

dissolved in 100 μ l of water to give final concentrations of 2 mM and 15 mM, respectively.

The quantitative determination of irreversible binding to microsomal macromolecules was performed as described earlier [6], except that the final protein pellet was dissolved in 0.5 ml Soluene-350 (Packard). A 0.2-ml aliquot of the protein solution was counted in 10 ml Dimilume-30 (Packard).

SDS-polyacrylamide gel electrophoresis was performed as described in detail elsewhere [8,9]. After incubation, 100 μ l aliquots of the incubation mixture (100–150 μ g of microsomal protein) were mixed with 100 μ l of a denaturing medium containing SDS [9] and boiled for 2 min. After cooling 100 μ l was applied to 11% SDS-polyacrylamide slab gels. The electrophoresis was run until the visible salt front reached the bottom of the gel. Gels were stained for 15 h in 0.1% Coomassie Brilliant Blue dissolved in 50% methanol and 7% acetic acid and destained electrophoretically in 25% methanol and 7% acetic acid. The gels were treated with PPO and dried [10] before fluorography. Kodak X-Omat films were exposed to the gels at -70°C for 1–6 weeks. Molecular weight standards used were bovine serum albumin ($M_r = 69\,000$), ovalbumin ($M_r = 45\,000$), soybean trypsin inhibitor ($M_r = 21\,500$) and sperm whale myoglobin ($M_r = 17\,200$).

RESULTS

Time-course and extent of metabolite binding to microsomes. When aromatic hydrocarbons are incubated with rat liver microsomes in the presence of an NADPH-generating system reactive metabolites are formed that will bind irreversibly to microsomal components. The time-dependent binding of phenol, BP and MC metabolites to microsomes from MC-induced rat livers was studied at a hydrocarbon concentration of 50 μM (Fig. 1). The extent of irreversible metabolite binding was the highest for phenol, and 2.8 nmol had bound per milligram membrane protein after 60 min of incubation corresponding to approx. 5% of the total amount of hydrocarbon added. BP and MC metabolites bound to a much smaller extent, 0.8 and 0.6 nmol/mg membrane protein corresponding to 1.5% and 1.2% of the added hydrocarbon. The binding of hydrocarbon metabolites to control and phenobarbital induced microsomes followed similar time-courses as in MC-induced microsomes. The extent of binding of MC and BP metabolites was lower, however, than in MC-induced microsomes (cf. Fig. 2). Phenol metabolite binding was less affected by inducers and the control microsomes even showed a higher binding than MC-induced microsomes (4 vs. 2.8 nmol/mg protein after 60 min of incubation). The extent of metabolite binding also appeared to be dependent on the substrate concentration used. More thorough studies on the quantitative aspects of metabolite binding are underway, where we examine the dependency on substrate concentration and the extent of metabolite binding to individual proteins.

The binding of all hydrocarbons examined was entirely dependent on the presence of an NADPH-generating system. It is clear therefore, that binding

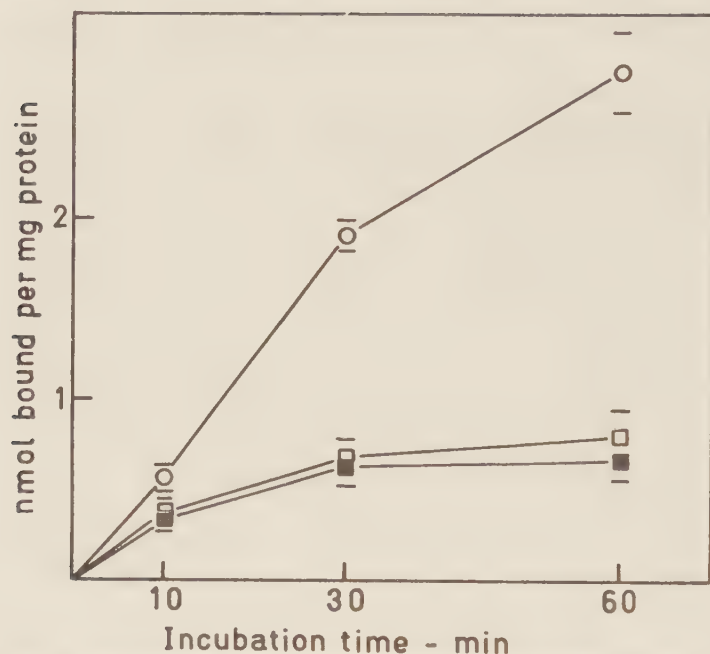
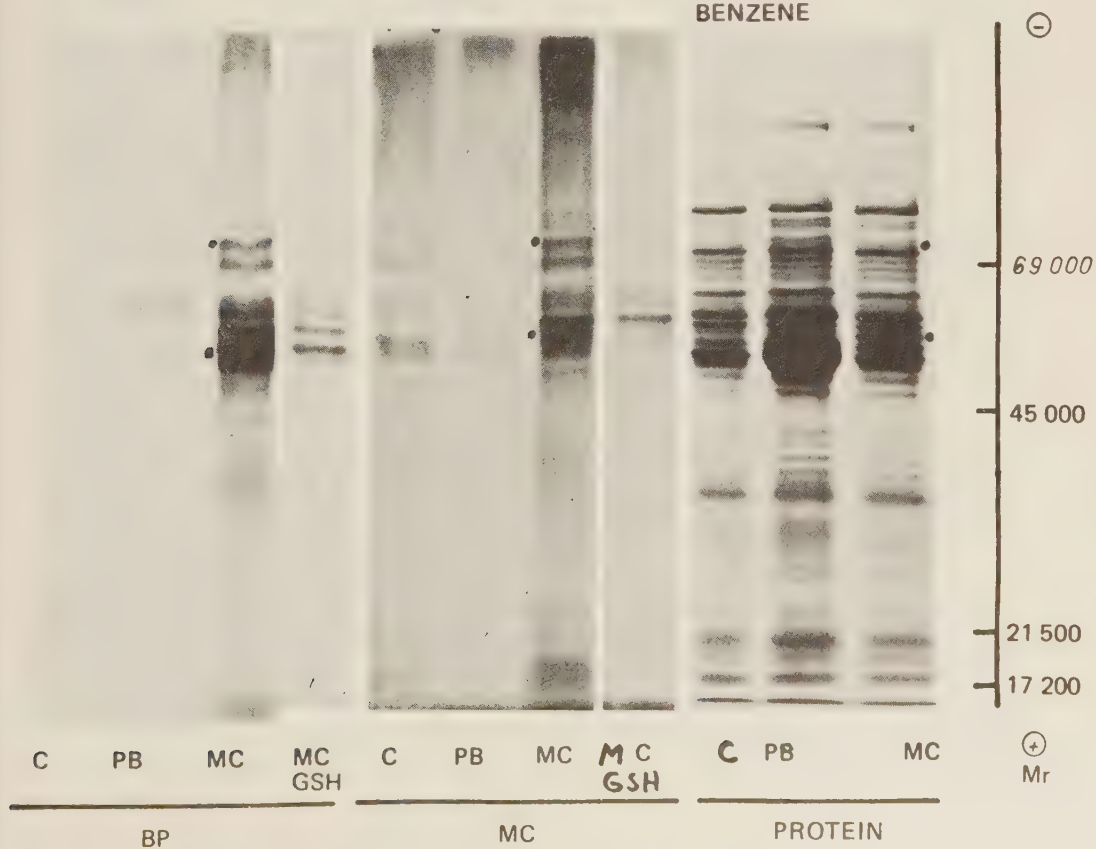
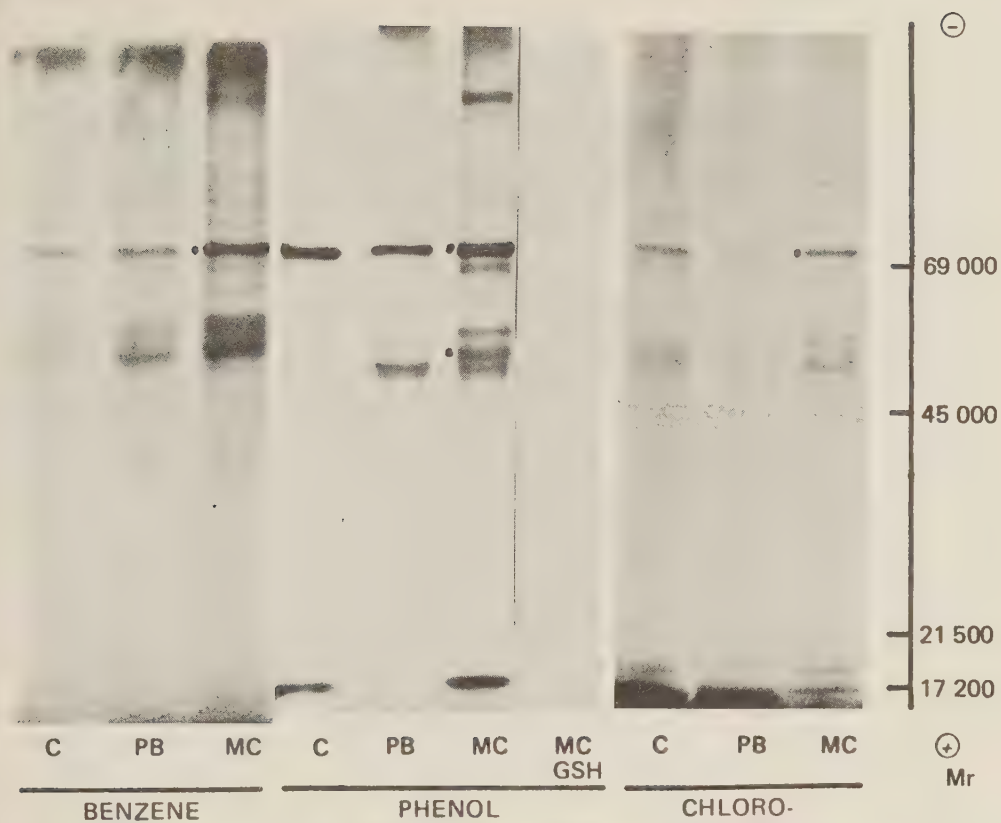


Fig. 1. Quantitative determination of the irreversible binding of metabolites of phenol (○), BP (□) and MC (■) to liver microsomes from MC-pretreated rats. The results are based on experiments with three animals and standard deviations are indicated. The amount of substrate was 60 nmol and the specific activities were for phenol 5.2 mCi/mmol, BP 13 mCi/mmol and MC 81 mCi/mmol. Microsomal protein (1.2 mg) was used in each incubation. Zero time incubations (<3% of the binding observed after 60 min) have been subtracted.

was preceded by a metabolic activation of the hydrocarbons to reactive metabolites.

Metabolite binding patterns to microsomal proteins. To examine whether the irreversible binding of metabolites to microsomal proteins occurred selectively or randomly, liver microsomes from control, phenobarbital and MC pretreated rats were incubated with radiolabelled aromatic hydrocarbons in the presence of an NADPH-generating system. The binding patterns of the metabolites were obtained by SDS-polyacrylamide gel electrophoresis and fluorography (Fig. 2).

Fig. 2. Hydrocarbon metabolite binding patterns and protein patterns of control and induced rat liver microsomes. Microsomes were incubated as described under Materials and Methods with one of the following hydrocarbons dissolved in 10 μ l acetone: [14 C]phenol, 13 nmol (35 mCi/mmol); [14 C]chlorobenzene, 417 nmol (5 mCi/mmol); [14 C]BP, 23 nmol (60.7 mCi/mmol); [3 H]MC, 0.92 nmol (9 Ci/mmol). After incubation samples were prepared for electrophoresis as described in Materials and Methods. C, PB and MC indicate control, phenobarbital- and methylcholanthrene-induced microsomes, respectively. The incubations contained 1.4 (C), 1.8 (PB) or 1.5 (MC) mg of microsomal protein. Metabolite binding patterns to MC-induced microsomes were also examined in presence of 2 mM GSH. The 56 000 and 72 000 M_r fractions specifically referred to in the text are indicated by dots.



The binding patterns of benzene, phenol and chlorobenzene metabolites were quite similar. Their most striking feature was the dominant radioactive band with an $M_r = 72\,000$ that was particularly conspicuous in the phenol incubations. Additional labelled bands occurred in the $50\,000$ – $60\,000\ M_r$ region. Phenol and chlorobenzene metabolites also bound extensively to a component in the high molecular weight region and to a component with an $M_r = 19\,000$. The relative density of the radioactive bands was affected by the microsome inducing system, with the most intense bands in the $50\,000$ – $60\,000\ M_r$ region occurring in the MC-induced microsomes.

The binding patterns for BP and MC metabolites were distinctly different from those of benzene, phenol and chlorobenzene. The enhancement in binding to MC-induced microsomes was particularly striking at the hydrocarbon concentrations used in this experiment. Most of the binding to these microsomes was concentrated in at least three bands in the $50\,000$ – $60\,000\ M_r$ region. One band at $68\,000\ M_r$ was rather prominent after incubation with BP and MC when compared to the other hydrocarbons. Two bands with $M_r\ 56\,000$ and $72\,000$ (indicated by dots in Fig. 2) were seen for all the hydrocarbons. The former occurred after MC-induction only and had the same mobility as a protein component induced by MC treatment which most likely is cytochrome *P-448* [11].

The presence of an NADPH-generating system was an absolute requirement for metabolite binding. When reduced glutathione was included in the incubation medium the binding of benzene, phenol and chlorobenzene metabolites was almost completely prevented (the inhibition was approx. 95%) while the binding of BP and MC metabolites was inhibited by around 65%.

The microsomal protein patterns are also shown in Fig. 2. The $56\,000\ M_r$ band and the position of the $72\,000\ M_r$ radioactive band have been marked by dots.

Effect of substrate concentration on metabolite binding patterns. The results presented in Fig. 2 were obtained with various hydrocarbon concentrations in the incubation medium. To examine the influence of substrate concentration on binding patterns microsomes from MC-induced rats were incubated with four concentrations of each of the substrates phenol, BP and MC. The metabolite binding patterns from this experiment are shown in Fig. 3. In the case of phenol most binding occurred to the $72\,000\ M_r$ component at the lowest substrate concentration tested. At higher phenol concentrations, however, this band was less dominant, and the major binding components in the $50\,000$ – $60\,000\ M_r$ region then showed a comparable extent of metabolite binding. The binding pattern of BP metabolites was not influenced significantly by the substrate concentration (there was a faint pattern at the highest concentration due to the low specific radioactivity that had to be used). The binding pattern of MC metabolites was dominated by the incorporation into the $56\,000\ M_r$ component at the lowest substrate concentration tested. At higher substrate concentrations, however, the incorporation occurred predominantly to two other components in the $50\,000$ – $60\,000\ M_r$ region.

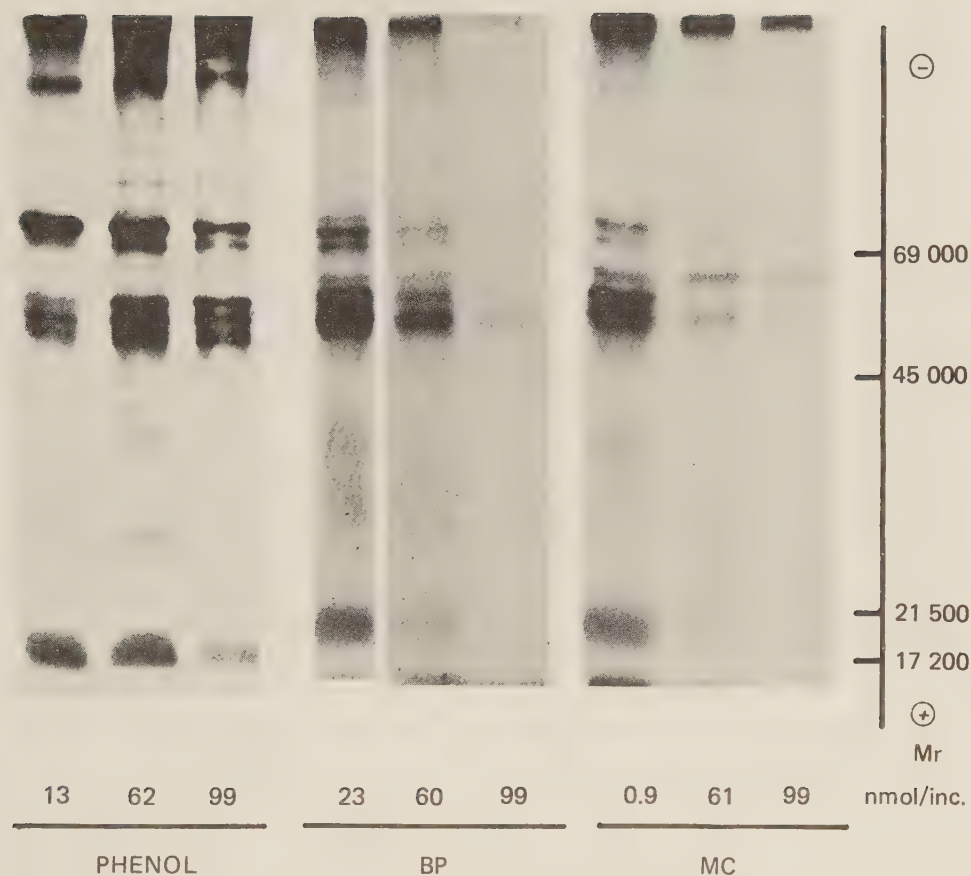


Fig. 3. Effect of substrate concentration on the binding patterns of phenol, BP and MC metabolites. Microsomes (1.4 mg) from MC-treated rats were incubated with the following amounts of substrate dissolved in 10 μ l acetone: phenol, 13 nmol (35 Ci/mmol), 62 nmol (7.6 mCi/mmol) and 99 nmol (4.7 mCi/mmol); BP, 23 nmol (61 mCi/mmol), 60 nmol (23 mCi/mmol) and 99 nmol (14 mCi/mmol); MC, 0.92 nmol (9 Ci/mmol), 61 nmol (136 mCi/mmol) and 99 nmol (84 mCi/mmol). After incubation electrophoresis and autoradiography were performed as described in Materials and Methods.

Effect of incubation time and UDPGA on metabolite binding patterns. The influence of incubation time on the binding patterns of phenol, BP and MC metabolites to liver microsomes from MC-induced rats was also examined (Fig. 4). The binding patterns for phenol and BP metabolites did not change appreciably with incubation time. In the case of MC metabolites, however, the band at 56 000 M_r was rather faint after 10-min incubation, but it became very prominent after 60 min. The presence of UDPGA, which will conjugate with metabolites containing hydroxy groups decreased the intensity of all bands in the BP and MC metabolite patterns (Fig. 4). This implies that at least a part of the binding to each band in the absence of

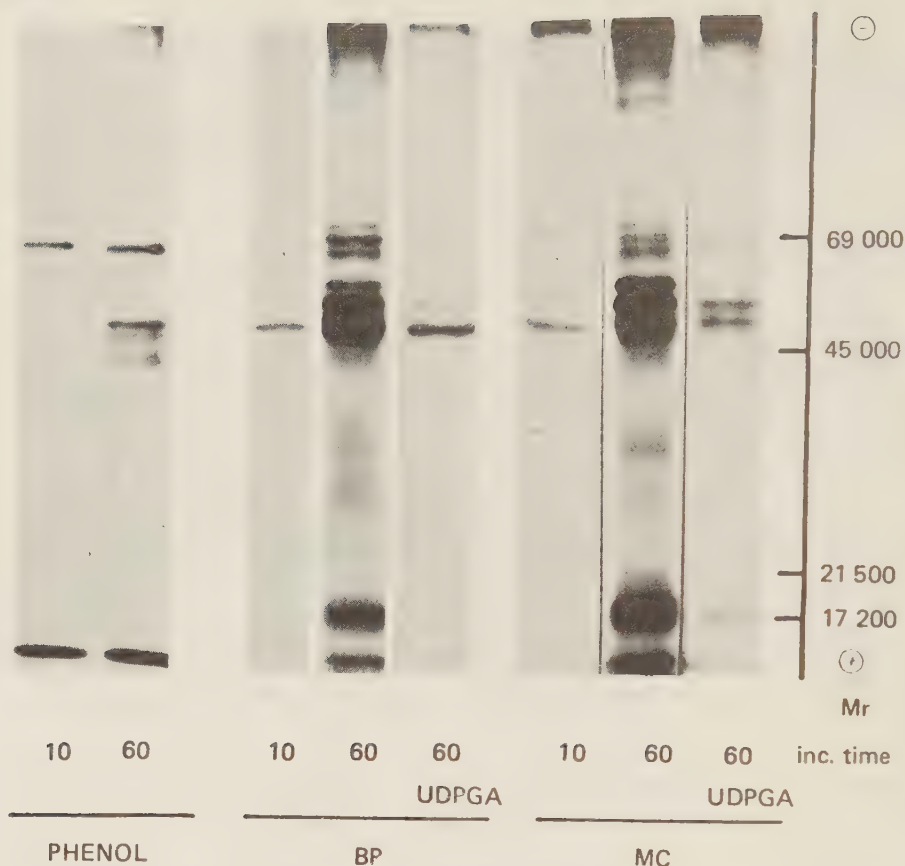


Fig. 4. Effect of incubation time and UDPGA on the binding patterns of phenol, BP and MC metabolites to MC-induced microsomes. The hydrocarbons were used at the lowest concentrations and corresponding specific radioactivities as given in the legend to Fig. 3. Incubation times were 10 min and 60 min. Microsomal protein (1.4 mg) was used in each incubation. UDPGA was used at a concentration of 15 mM. After incubation electrophoresis and autoradiography were performed as described in Materials and Methods.

UDPGA was due to secondary metabolites. The binding of phenol metabolites in the presence of UDPGA was not tested since phenol itself would conjugate with this compound.

DISCUSSION

The potential importance of hydrocarbon metabolite binding to protein for the generation of cell toxicity has been recognized for several years [4]. As far as we know, however, a direct link between metabolite binding to specific proteins in the cell and cell toxicity has not been established.

Our results show that in isolated microsomes there is a preferential

binding of hydrocarbon metabolites to a small set of proteins. It is possible, therefore, that if there is a link between metabolite binding and cell toxicity, this might be due to specific rather than random interactions between metabolites and proteins.

In order to bind to proteins the toxic and/or carcinogenic hydrocarbons tested had to be metabolically activated as indicated by the absolute requirement of an NADPH-generating system for binding. The activation produces electrophilic intermediates which then react with nucleophilic sites in macromolecules [12–14]. Nucleophilic binding sites in proteins include the sulfhydryl group of cysteine. It has been shown earlier that metabolically activated MC and 7,12-dimethylbenzanthracene most likely bind to proteins via such sulfhydryl groups [15]. A similar type of binding, at least for the major part of the ultimate binding metabolites of the hydrocarbons examined here, is suggested by the inhibition of binding by reduced glutathione.

The most extensive binding was observed for phenol metabolites of which more than 2.5 nmol bound irreversibly per milligram of microsomal protein. Since the binding occurred preferentially to one protein fraction ($M_r = 72\,000$), several phenol metabolites appear to have bound to each protein molecule. An exact determination of the binding stoichiometry has to await the isolation of the binding protein. The experiments with increasing concentrations of phenol in the incubation medium or longer incubation times showed that other protein components also would bind phenol metabolites under such conditions. It is likely that the potential binding sites of the 72 000 M_r protein then became saturated and that excess metabolites reacted with proteins which were normally less prone to bind metabolites. Metabolites of the other hydrocarbons bound to a much smaller extent and no saturation was observed in these cases.

Since the hydrocarbon metabolites appeared to bind specifically rather than randomly to microsomal proteins and since different protein binding patterns were observed for different hydrocarbons the question arises which factors will determine the specificity of metabolite binding. One possibility is that the kind of ultimate binding metabolite formed (for instance a semi-quinone or an epoxide) determines the specific target protein(s). Another possibility is that the metabolite will bind to any macromolecule with an exposed reactive group. The latter suggestion implies that any newly formed reactive metabolite most likely will bind to a reactive protein close to the site of metabolite formation. Thus, the enzyme that catalyses the formation of the metabolite would itself receive most of the binding provided it contains an exposed reactive group. A matter in point is the radioactive band of 56 000 M_r which is obtained after incubation of all five hydrocarbons with MC-induced microsomes. This band has the same mobility in the gel as a protein fraction induced by MC which is likely to be cytochrome *P-448*. However, it is not possible from this observation alone to resolve which factor determines the specificity of metabolite binding, particularly since the complete metabolic conversion pathways for the various hydrocarbons remain unclear.

Either of the suggested mechanisms for binding specificity permits the interpretation that the similarities in metabolite binding patterns for benzene, phenol and chlorobenzene depend on similar activation pathways for these compounds. The ultimate binding metabolites for phenol and benzene are probably mainly quinones and semiquinones [6; A. Tunek, K.L. Platt and F. Oesch, unpublished). Chloro- and bromobenzene have both been thought to be activated by the mixed-function oxidase system to epoxides which would bind to macromolecules causing hepatotoxic effects [16–18]. Provided that there is a correlation between the type of ultimate binding metabolite and binding pattern, our results agree more with the recent suggestion [19], that at least a part of the protein binding metabolites of bromobenzene originate through a further metabolism of the hydroxylated compound to a semiquinone or a quinone.

The metabolic activation of BP and the subsequent binding to DNA has been studied in great detail. Dihydrodiol epoxides and further oxidized BP-phenols (mainly BP-9-hydroxy-4,5-oxide) seem to be the main binding metabolites produced by MC-induced microsomes [20,21]. Less is known about the metabolic activation of MC. Recent data indicate, however, that several dihydrodiol epoxides can be formed [22,23]. The binding patterns for BP and MC were rather similar, but they were distinctly different from those of benzene, phenol and chlorobenzene. Thus, the available data on the activation pathways of the compounds examined and the binding patterns presented here suggest that differences in the mechanism of metabolic activation also are reflected in differences in binding patterns.

The influence of substrate concentration on the binding pattern of MC-metabolites is interesting. At the lowest concentration used a band at 56 000 M_r is clearly dominating, but at higher MC-concentrations this band is very faint. Figure 4 shows the influence of incubation time on the binding patterns of phenol, BP and MC-metabolites at a substrate concentration identical to the lowest one in Fig. 3. Here the metabolite pattern is dominated by the 56 000 M_r band only after the longest incubation time. These observations suggest that the binding of MC metabolites to the 56 000 M_r protein may require more metabolic steps than the binding to other proteins.

In summary, the findings presented in this paper suggest that there are differences in binding patterns of activated hydrocarbons to microsomal proteins and that the pattern is dependent on the type of metabolite which is the ultimate binding agent. Of interest may now be to isolate and identify metabolite target proteins in order to examine the influence of metabolite binding on their biological function and the possible role of binding for the development of cytotoxicity.

ACKNOWLEDGEMENTS

We thank Drs. Franz Oesch and Cecil and Michèle Fox for valuable discussions and Thomas Henriksson for the photographical work. We

acknowledge the support by grants from the Swedish Cancer Society and the Swedish Natural Science Research Council.

REFERENCES

- 1 D.J. Jollow, J.J. Kocsis, R. Snyder and H. Vainio (Eds.), *Biological reactive intermediates. Formation, toxicity and inactivation*, Plenum Press, New York, 1977.
- 2 E.C. Miller and J.A. Miller, *Biochemical mechanisms of chemical carcinogenesis*, in: H. Busch (Ed.), *Molecular Biology of Cancer*, Academic Press, New York, 1972, p. 377.
- 3 B.B. Brodie, *Idiosyncrasy and intolerance*, in: G.E.W. Wolstenholme and R. Porter (Eds.), *CIBA Foundation Symposium on Drug Responses in Man*, Churchill, London, 1967, p. 188.
- 4 H. Uehleke, *The model system of microsomal drug activation and covalent binding to endoplasmic proteins*, *Excerpta Medica*, 15 (1974) 119.
- 5 C. Wyndham, J. Devenish and S. Safe, *The in vitro metabolism, macromolecular binding and bacterial mutagenicity of 4-chlorobiphenyl, a model PCB substrate*, *Res. Commun. Chem. Pathol. Pharmacol.*, 15 (1976) 563.
- 6 A. Tunek, K.L. Platt, P. Bentley and F. Oesch, *Microsomal metabolism of benzene to species irreversibly binding to microsomal protein and effects of modifications of this metabolism*, *Mol. Pharmacol.*, 14 (1978) 920.
- 7 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.T. Randall, *Protein measurement with the Folin phenol reagent*, *J. Biol. Chem.*, 193 (1951) 265.
- 8 D.M. Neville, Jr., *Molecular weight determination of proteindodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system*, *J. Biol. Chem.*, 246 (1971) 6328.
- 9 M. Sommarin, R. Ohlsson, T. Henriksson, C. Schelin and B. Jergil, *Protein phosphorylation in subfractions of rat liver endoplasmic reticulum*, submitted for publication.
- 10 W.M. Bonner and R.A. Laskey, *A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels*, *Eur. J. Biochem.*, 46 (1974) 83.
- 11 A.F. Welton and S.D. Aust, *Multiplicity of cytochrome P₄₅₀ hemoproteins in rat liver microsomes*, *Biochem. Biophys. Res. Commun.*, 56 (1974) 898.
- 12 J.R. Gillette, J.R. Mitchell and B.B. Brodie, *Biochemical mechanisms of drug toxicity*, *Annu. Rev. Pharmacol.*, 14 (1974) 271.
- 13 D.M. Jerina and J.W. Daly, *Arene oxides: a new aspect of drug metabolism*, *Science*, 185 (1974) 573.
- 14 F. Oesch, *Mammalian epoxide hydrases: inducible enzymes catalyzing the inactivation of carcinogenic and cytotoxic metabolites derived from aromatic and olefinic compounds*, *Xenobiotica*, 3 (1973) 305.
- 15 T.H. Corbett and P. Nettesheim, *Separation of multiple forms of protein-bound metabolites of the carcinogenic hydrocarbons 3-methylcholanthrene and 7,12-dimethylbenz(a)anthracene from several rodent species*, *Chem.-Biol. Interact.*, 8 (1974) 285.
- 16 D.J. Jollow, J.R. Mitchell, N. Zampaglione and J.R. Gillette, *Bromobenzene-induced liver necrosis. Protective role of glutathione and evidence for 3,4-bromobenzene oxide as the hepatotoxic metabolite*, *Pharmacology*, 11 (1974) 151.
- 17 H.G. Selander, D.M. Jerina, D.E. Piccolo and G.A. Berchtold, *Synthesis of 3- and 4-chlorobenzene oxides. Unexpected trapping results during metabolism of [¹⁴C]-chlorobenzene by hepatic microsomes*, *J. Am. Chem. Soc.*, 97 (1975) 4428.
- 18 N. Zampaglione, D.J. Jollow, J.R. Mitchell, B. Stripp, M. Hamrick and J.R. Gillette, *Role of detoxifying enzymes in bromobenzene-induced liver necrosis*, *J. Pharmacol. Exp. Ther.*, 187 (1973) 218.

- 19 S. Hesse, T. Wolff and M. Mezger, Irreversible binding of aromatic hydrocarbons catalyzed by microsomes: involvement of phenolic metabolites, 6th European Workshop on Drug Metabolism, Leiden, (1978) abstract No. 161.
- 20 O. Pelkonen, A.R. Boobis, H. Yagi, D.M. Jerina and D.W. Nebert, Tentative identification of benzo(a)pyrene metabolite-nucleoside complexes produced in vitro by mouse liver microsomes, *Mol. Pharmacol.* 14 (1978) 306.
- 21 B. Jernström, S. Orrenius, O. Undeman, A. Gräslund and A. Ehrenberg, Fluorescence study of DNA-binding metabolites of benzo(a)pyrene formed in hepatocytes isolated from 3-methylcholanthrene-treated rats, *Cancer Res.*, 38 (1978) 2600.
- 22 T.A. Stoming, W. Bornstein and E. Bresnick, The metabolism of 3-methylcholanthrene by rat liver microsomes — A reinvestigation, *Biochem. Biophys. Res. Commun.*, 79 (1977) 461.
- 23 A.W. Wood, R.L. Chang, W. Levin, P.E. Thomas, D. Ryan, T.A. Stoming, D.R. Thakker, D.M. Jerina and A.H. Conney, Metabolic activation of 3-methylcholanthrene and its metabolites to products mutagenic to bacterial and mammalian cells, *Cancer Res.*, 38 (1978) 3398.

Toxic Effects of Benzene and Benzene Metabolites on Granulopoietic
Stem Cells and Bone Marrow Cellularity in Mice. ¹

A. Tunek, T. Olofsson ^a and M. Berlin.

Institute of Environmental Health, University of Lund, Sölvegatan 21,
S-223 62 LUND, Sweden, and ^a Research Department 2, E-blocket,
University Hospital, S-221 85 LUND, Sweden.

Running title:

Mechanism of benzene toxicity.

ABSTRACT

Toxic Effects of Benzene and Benzene Metabolites on Granulopoietic Stem Cells and Bone Marrow Cellularity in Mice. Tunek, A., Olofsson, T. and Berlin, M. (1980). Toxicol. Appl. Pharmacol.

Mice were injected subcutaneously with benzene or one of three of its metabolites (phenol, hydroquinone or 1,2-dihydro-1,2-dihydroxy benzene). The adverse effects on the concentration of granulopoietic stem cells (measured as number of colony forming units per tibia or per 10^5 cells) and on the bone marrow cellularity in tibia were measured. Benzene had strong toxic effects. Thus, 0.7 mg benzene/kg body weight injected daily on 6 consecutive days gave detectable effects on stem cell concentration, and 3.5 mg/kg/day affected also cellularity. Six daily injections of 440 mg benzene/kg reduced cellularity and number of colony forming units per tibia by 86-95%. None of the benzene metabolites tested could reproduce the strong effects of benzene when injected subcutaneously, although phenol significantly affected stem cell concentration. Toluene, a competitive inhibitor of benzene metabolism, significantly alleviated the effects of benzene, and pretreatment of the mice with phenobarbital also slightly reduced the effects. Regeneration of the bone marrow after benzene injections occurred rapidly during the first week, then at a slower rate for the next four weeks. At this time cellularity and granulopoietic stem cell concentration were restored, but the fraction of stem cells in S-phase was still higher than in controls, indicating a still elevated proliferation rate.

INTRODUCTION

It is well established that the blood forming elements are particularly susceptible to the toxicity of benzene. In humans, who have been occupationally exposed to benzene, reduction in the number of circulating white blood cells, chromosome aberrations in white blood cells, bone marrow damage and leukemia have been described (for a review see Goldstein, 1977).

It was early shown that bone marrow cells of virtually all cell lines were affected following exposure to benzene (Selling, 1916). Chromosome breaks and gaps (Forni and Moreo, 1967; Fredga et al., 1979), micronuclei in erythrocyte precursors (Siou and Conan, 1977; Hite et al., 1980), changes in the relative populations of several cell lines (Koike et al., 1959) and inhibition of incorporation of Fe into erythrocyte precursors (Lee et al., 1973) are among the cytological changes induced by benzene. Some studies indicate that the fully differentiated blood cells and the multipotent stem cells are relatively resistant to the toxic actions of benzene (Steinberg, 1949; Lee et al., 1974; Irons et al., 1979). Instead, bone marrow cells in the maturation and proliferation process are the targets for benzene toxicity.

Although it is generally assumed that metabolic activation is necessary for the development of benzene toxicity, data in the literature are often apparently conflicting, and show that this question is very complex. Pretreatment of rats with phenobarbital, an inducer of benzene metabolism at least in the liver, made the animals more resistant to the leukopenic actions of benzene (Ikeda and Ohtsuji, 1971; Gill et al., 1979). On the other hand, pretreatment of rats with 3-methylcholanthrene, which also induces benzene metabolism, or SKF-525A², which inhibits this metabolism, did not affect the degree of benzene-induced leukopenia (Gill et al., 1979). Simultaneous administration of benzene and toluene, a competitive inhibitor of benzene metabolism, gave a smaller adverse effect on incorporation of Fe into erythrocytes than did benzene alone (Andrews et al., 1977). Attempts have also been made to test

several benzene metabolites for their potential as hemotoxic agents. Of phenol, catechol, hydroquinone, hydroxyhydroquinone, trans-trans-muconic acid, D,L-phenylmercapturic acid and potassium phenylsulphate, only catechol appeared to depress bone marrow function by causing anemia and leukopenia (Nomiya, 1965). Other researchers, however, failed to reproduce these results; catechol and hydroquinone, at doses killing 50% of the animals, did not produce hemotoxic effects (Mitchell, 1971). In cultures of human lymphocytes, benzene was shown not to induce sister chromatid exchanges, while phenol and, particularly, catechol and hydroquinone were active (Morimoto and Wolff, 1980).

Recent research on the metabolic activation of benzene in rat liver microsomes indicated that benzene oxide was formed as a metabolite of benzene, but that quinones and/or semiquinones contributed considerably more to the observed covalent binding to microsomal proteins (Tunek et al., 1978; Tunek et al., in press).

In the present study we have investigated the toxic effects of benzene and some of its metabolites on bone marrow cellularity and on the progenitors of the white blood cells, i.e. the granulopoietic stem cells in mice. The method to study the granulopoietic stem cells is based on the ability of these cells to form colonies in culture after stimulation with "colony stimulating factors" (Metcalf, 1973). The cells proliferating to form such colonies in culture are termed CFU-C². Benzene has been shown to be toxic in a similar system, but only high doses were used, and no attempts were made to elucidate the actions of any metabolites (Uyeki et al., 1977). In addition, we have used the "³H-thymidine suicide" method to determine the influence of benzene on the fraction of CFU-C present in the S-phase. The size of this fraction can be taken as a measure of the proliferation rate (Olofsson and Olsson, 1976).

METHODS

Injection of benzene and its metabolites

NMRI mice weighing about 20 g were injected subcutaneously once daily with benzene or its metabolites and sacrificed by cervical dislocation 16-20 h after the last injection. Four or eight animals were used in each group. Doses and number of injections are indicated in each case. Benzene and toluene were diluted in olive oil (0.1 ml each injection) and the water soluble metabolites of benzene, i.e. phenol, hydroquinone and benzene dihydrodiol², were dissolved in physiological saline (0.1 ml each injection). Benzene, toluene and hydroquinone were of analytical grade and purchased from Merck. Benzene dihydrodiol, synthesized as described earlier (Platt and Oesch, 1977), was kindly supplied by drs. Karl Platt and Franz Oesch, Mainz, Germany. All animals used in the experiments received identical amounts of olive oil (6 x 0.1 ml), since we found that the oil itself had a tendency to increase the cellularity of the bone marrow. Saline was given only in combination with the water soluble metabolites, as we found that saline alone did not affect the parameters measured. Phenobarbital was given in the drinking water (0.1%) from the day before starting the treatments till the animals were sacrificed.

Bone marrow culture

The marrow from one tibia was collected by flushing the marrow cavity with 1.5-2 ml of McCoy's medium. The marrows from 4-8 mice were pooled and gently suspended with Pasteur pipette. 1×10^5 cells were cultured in 1.0 ml of McCoy's medium with 15% fetal calf serum and 10% colony stimulating activity prepared from extracts of mouse embryo (Bradley et al., 1971), in 0.3% agar on top of 1.0 ml 0.5% agar in McCoy's medium with 15% fetal calf serum in 35 mm petri dishes (Falcon, Los Angeles, USA). After 7 days of incubation in 5% CO₂ in fully humidified atmosphere at 37°C colonies (>50 cells/aggregate) were counted in an inverted microscope. Four replicates were used in all tests. Results are given as the number of CFU-C

per tibia or per 10^5 cells, or as the number of nucleated cells per tibia. Student's t-test was used in the statistical analysis.

For determination of the fraction of CFU-C in S-phase the " ^3H -thymidine suicide" method was used (Olofsson and Olsson, 1976): to one of two tubes containing 1.5×10^6 marrow cells in 0.75 ml McCoy's medium with 1% fetal calf serum 20 μCi ^3H -thymidine (17-21 Ci/mmol; Amersham, England) was added and the tubes incubated at 37°C for 30 min. The cells were then washed once in 5 ml of McCoy's medium with 100 μg cold thymidine/ml and once in 5 ml McCoy's medium with 1% fetal calf serum and cultured in agar as described. The reduction of colony forming cells on incubation with ^3H -thymidine is taken as measure of the fraction of CFU-C in S-phase.

RESULTS

Dose-response relationships

Mice were injected with benzene in daily doses of 0.7, 3.5, 18, 88 and 440 mg/kg body weight on 6 consecutive days and sacrificed on day 7. Results are shown in Fig. 1. There was a significant reduction of CFU-C/ 10^5 cells already with the lowest dose ($p < 0.01$). CFU-C/tibia followed a similar curve with significant reduction at 3.5 mg benzene/kg and at higher doses ($p < 0.001$). Cellularity was also reduced at 3.5 mg/kg, but a dramatic effect was seen only at the highest dose.

In preliminary experiments we had noticed that single injections of benzene did not always affect the bone marrow cellularity and stem cell concentration. Mice were therefore injected with benzene in 1-6 daily doses of 440 mg/kg and sacrificed on the day after the last benzene injection (all animals received 6 doses of olive oil). These results are shown in Fig. 2. Maximal effect was achieved with 4-6 daily benzene injections, which reduced cellularity and CFU-C/tibia by 86-95% and CFU-C/ 10^5 cells by 32-56%. In subsequent experiments 6 daily injections were used.

Effect of benzene metabolites

We also investigated the effects of some of the metabolites of benzene to see if we could achieve the same reduction of cellularity and stem cell concentration as with benzene itself. To cover the major pathways of benzene metabolism phenol, hydroquinone and benzene dihydrodiol were chosen. The results are given in Table 1. Phenol had no effect on cellularity but significantly reduced the number of CFU-C/tibia ($p < 0.05$) and CFU-C/ 10^5 cells ($0.05 > p < 0.001$) at a dose of 6×50 mg/kg. Hydroquinone had no effect on CFU-C/tibia or cellularity but showed a tendency to increase the number of CFU-C/ 10^5 cells at 6×50 mg/kg. Benzene dihydrodiol had only slightly reducing effects on cellularity and the number of CFU-C/tibia in one of two experiments at 6×50 mg/kg.

The amount of subcutaneously injected benzene, giving rise to 50 mg phenol/kg body weight in mice, would be in the order of 100 mg/kg (calculation based on information given by Andrews et al., 1977). Fig. 1 shows that this dose of benzene gave much stronger effects than did the phenol (Table 1). Benzene dihydrodiol and hydroquinone are quantitatively minor metabolites of benzene (Rusch et al., 1977). The amounts injected in these experiments several-fold exceed the amounts expected to be formed from 440 mg benzene/kg body weight. Thus, none of the metabolites tested reproduced the strong toxic effects of benzene when injected subcutaneously.

Effect of toluene on benzene toxicity

The effects of toluene, a competitive inhibitor of benzene metabolism (Andrews et al., 1977) were studied with and without the simultaneous administration of benzene. The results are shown in Table 2. Toluene itself did not show the dramatic effect of benzene even at a dose of 6×867 mg/kg body weight, although some reduction of cellularity and number of CFU-C was evident in one experiment. When toluene and benzene were given together, toluene significantly alleviated the effects of benzene on both cellularity and number of CFU-C/tibia ($p < 0.001$).

Effect of phenobarbital pretreatment on benzene toxicity

Phenobarbital was given to induce an increased metabolism of benzene in the liver. Phenobarbital itself had somewhat variable effects on the marrow cells (Table 3). When given together with benzene, however, phenobarbital showed a tendency to reduce the toxic effects of benzene, especially the suppression of CFU-C/tibia.

Bone marrow regeneration after benzene injections

Benzene was given in 6 daily injections (440 mg/kg) and the regeneration of the bone marrow was followed over a period of five weeks. The results are shown in Fig. 3. Reconstitution of cellularity and CFU-C/tibia started two days after the last injection and showed a rapid initial phase between days 2 and 8. A second slower phase of regeneration then followed with steadily increasing numbers of CFU-C/tibia over the next four weeks, whereas cellularity showed a rebound decrease during the second week before paralleling the recovery of CFU-C. After five weeks cellularity and number of CFU-C/tibia were restored to normal values. The fraction of CFU-C in S-phase showed very high values and oscillated during the regeneration period. At five weeks the fraction of CFU-C in S-phase was still higher than normal (normal mean: $42.2\% \pm 2.3$ SD., $n = 4$).

DISCUSSION

In this report we describe the suppression of bone marrow cellularity and marrow granulopoietic stem cells by subcutaneous administration of benzene to mice. At low benzene doses the number of CFU-C/tibia was considerably more affected than was the overall cellularity. Thus, the granulopoietic stem cells appear to be sensitive targets for benzene toxicity. Furthermore, our data indicate that fractionation of the dose over several days is more effective than the same dose given in a single injection, since 3.5 mg/kg injected 6 times gave a comparable effect to 440 mg/kg injected once. It can be calculated that a mouse would retain about 3.5 mg benzene/kg if exposed to a benzene atmosphere of 4.0 ppm for 8 h. This calculation

is based on 0.023 l/min as respiration rate for a 20 g mouse (Guyton, 1947) and on the assumption that the retention of inhaled benzene in the mouse is similar to that in man, namely about 50% (Hunter, 1966). The parameters studied thus seem to be highly sensitive to benzene.

The regeneration of marrow cellularity and stem cell content after benzene exposure started promptly and was rapid during the first week and then continued at a slower rate over the next four weeks. At this time marrow cellularity and stem cell content were restored to normal levels but the still elevated fraction of CFU-C in S-phase, an expression of an accelerated proliferation rate, indicated that regeneration was not fully completed.

It is generally assumed that benzene requires metabolic activation to exert its toxic effects (for a review see Snyder and Kocsis, 1975). The liver is probably the major organ for metabolism of benzene, but it has also been shown that benzene can be metabolized within the bone marrow (Andrews et al., 1979). The hemotoxic effects of benzene are thus most likely related to metabolites produced in the marrow or in the liver. The latter possibility gains some support from the fact that partial hepatectomy reduces the toxic effects of benzene (Mitchell, 1971; Sammett et al., 1979). Recent research indicated that p-benzoquinone or p-benzosemiquinone, rather than benzene oxide, are the metabolites of benzene causing covalent binding to macromolecules in microsomal incubations (Tunek et al., 1978; Tunek et al., in press). These metabolites are formed in a multi-step pathway including phenol and hydroquinone as intermediates. Another metabolite of benzene is the benzene dihydrodiol (Sato et al., 1963) that eventually could give rise to catechol, o-benzoquinone, o-benzosemiquinone or a dihydrodiol epoxide. We therefore investigated the effects of phenol, hydroquinone and benzene dihydrodiol. Phenol produced some inhibition of CFU-C/ 10^5 cells and CFU-C/tibia but the effect was weaker than that of benzene. Hydroquinone gave no significant reduction and benzene dihydrodiol showed only minor toxic effects.

Toluene is a substrate for cytochrome P-450 and has been shown to

competitively inhibit benzene metabolism (Andrews et al., 1977). When toluene was given simultaneously with benzene, the toxic effects of benzene were significantly alleviated. This phenomenon has been observed by others in other biological systems and has been taken as an indication that benzene metabolites rather than benzene itself exert the toxic effects.

Phenobarbital has been used extensively as an enzyme inducer to enhance drug metabolism primarily in the liver (Conney, 1967) and is known to induce benzene metabolism in liver microsomes (Snyder et al., 1967; Tunek and Oesch, 1979). The dose of liver metabolites reaching the bone marrow should be a function of blood concentrations of these metabolites. The blood levels have rarely been measured, but it can be assumed that those levels are reflected by urinary excretion of metabolites. There are conflicting reports, however, as to how phenobarbital pretreatment influences urinary excretion of benzene metabolites after subcutaneous injections of benzene. In one study a clear increase in urinary excretion of free and conjugated phenol was observed (Gill et al., 1979), while in another report no significant increase could be demonstrated despite the fact that $v_{\max}$ of the hepatic microsomal hydroxylation of benzene was elevated 6-fold (Gut, 1976).

In our study, phenobarbital pretreatment did in fact slightly reduce the toxic effects of benzene on the marrow cells. A possible explanation for this is that an increased metabolism in the liver might have protected the bone marrow from some of the benzene injected, but it may also imply that whatever metabolite is important for marrow toxicity, that metabolite probably has to be formed within the bone marrow to be fully active. This interpretation is also consistent with the mild effects of the benzene metabolites on subcutaneous injections described in this report.

We show in this paper that phenol, hydroquinone and benzene dihydrodiol do not reproduce the strong toxic effect of benzene when injected subcutaneously. At the same time metabolic activation appears to be a prerequisite for the toxicity. It is therefore

tempting to regard the data as support for the concept that benzene oxide is the ultimate toxic metabolite of benzene. Although this might be correct, it would in our opinion be premature to interpret the data in that way. It is possible that these hydroxylated metabolites are so effectively conjugated in the liver and in other organs that very little will reach the bone marrow. Thus, it could be that the compounds have to be formed in the bone marrow or be applied more directly to this organ to be able to exert their toxic effects. We feel, therefore, that it is of great importance to study the effects of benzene and its metabolites directly on the bone marrow cells to further elucidate the mechanism of action of benzene. A culture system for mouse marrow cells in vitro, capable of regenerating multipotent and committed stem cells over periods of many weeks, has been designed by Dexter et al. (1977). This culture system opens the possibility to study the effects of benzene and its metabolites directly on the production of hemopoietic stem cells, circumventing any unresolved problems of resorption and intermediate metabolism. Such studies are now being performed in our laboratory. Recently, induction of leukemia with Friend and Abelson virus in such long term cultures of mouse marrow in vitro was achieved, which demonstrates the potency of this experimental model (Greenberger et al., 1979).

ACKNOWLEDGEMENT

The benzene dihydrodiol used in this study was a generous gift from
drs. Karl Platt and Franz Oesch, Mainz, Germany.

REFERENCES

- Andrews, L.S., Lee, E.W., Witmer, C.M., Kocsis, J.J. and Snyder, R. (1977). Effects of toluene on the metabolism, disposition and hemopoietic toxicity of ^3H -benzene. Biochem. Pharmacol. 26, 293-300.
- Andrews, L.S., Sasame, H.A. and Gillette, J.R. (1979). ^3H -Benzene metabolism in rabbit bone marrow. Life Sci. 25, 567-572.
- Bradley, T.R., Stanley, E.R. and Sumner, M.A. (1971). Factors from mouse tissues stimulating colony growth of mouse bone marrow cells in vitro. Aust. J. Exp. Biol. Med. Sci. 49, 595-603.
- Conney, A.H. (1967). Pharmacological implications of microsomal enzyme induction. Pharmacol. Rev. 19, 317-366.
- Dexter, T.M., Allen, T.D. and Lajtha, L.G. (1977). Conditions controlling the proliferation of haemopoietic stem cells in vitro. J. Cell Physiol. 91, 335-344.
- Forni, A. and Moreo, L. (1967). Cytogenetic studies in a case of benzene leukemia. Eur. J. Cancer 3, 251-255.
- Fredga, K., Reitalu, J. and Berlin, M. (1979). Chromosome studies in workers exposed to benzene. In: Genetic Damage in Man Caused by Environmental Agents (E.K. Berg, ed.), pp. 187-203, Academic Press, New York.
- Gill, D.P., Kempen, R.R., Nash, J.B. and Ellis, S. (1979). Modifications of benzene myelotoxicity and metabolism by phenobarbital, SKF-525A and 3-methylcholanthrene. Life Sci. 25, 1633-1640.
- Goldstein, B.D. (1977). Hematotoxicity in humans. J. Tox. Env. Health suppl. 2, 69-106.

- Greenberger, J.S., Davisson, P.B., Gans, P.J. and Moloney, W.C. (1979). In vitro induction of continuous acute promyelocytic leukemia cell lines by Friend and Abelson leukemia virus. Blood 53, 987-1001.
- Gut, I. (1976). Effect of phenobarbital pretreatment on in vitro enzyme kinetics and in vivo biotransformation of benzene in the rat. Arch. Toxicol. 35, 195-206.
- Guyton, A.C. (1947). Measurement of the respiratory volumes of laboratory animals. Am. J. Physiol. 150, 70-77.
- Hite, M., Pecharo, M., Smith, I. and Thornton, S. (1980). The effect of benzene in the micronucleus test. Mut. Res. 77, 149-155.
- Hunter, C.G. (1966). Aromatic solvents. Ann. Occup. Hyg. 9, 191-197.
- Ikeda, M. and Ohtsuji, H. (1971). Phenobarbital-induced protection against toxicity of toluene and benzene in the rat. Toxicol. Appl. Pharmacol. 20, 30-43.
- Irons, R.D., Heck, H. d'A., Moore, B.J. and Muirhead, K.A. (1979). Effects of short-term benzene administration on bone marrow cell cycle kinetics in the rat. Toxicol. Appl. Pharmacol. 51, 399-409.
- Koike, S., Kawai, K. and Sugimoto, H. (1959). Experimental studies on benzene poisoning. Effect of benzene on the blood and bone marrow in albino rats. Bull. Nat. Inst. Ind. Health 2, 1-16.
- Lee, E.W., Kocsis, J. and Snyder, R. (1973). Dose dependent inhibition of ⁵⁹Fe incorporation into erythrocytes after a single dose of benzene. Res. Commun. Chem. Pathol. Pharmacol. 5, 547-550.
- Lee, E.W., Kocsis, J.J. and Snyder, R. (1974). Acute effects of benzene on ⁵⁹Fe incorporation into circulating erythrocytes. Toxicol. Appl. Pharmacol. 27, 431-436.

- Metcalf, D. (1973). Regulation of granulocyte and monocyte-macrophage proliferation by colony stimulating factor (CSF): a review. Exp. Hemat. 1, 185-201.
- Mitchell, J.R. (1971). Mechanism of benzene-induced aplastic anemia. Fed. Proc. 30, 2044 (abstract).
- Morimoto, K. and Wolff, S. (1980). Increase of sister chromatid exchanges and perturbations of cell division kinetics in human lymphocytes by benzene metabolites. Cancer Res. 40, 1189-1193.
- Nomiyama, K. (1965). Studies on poisoning by benzene and its homologues. 7. Toxicity of benzene metabolites to hemopoiesis. Ind. Health 3, 53-57.
- Olofsson, T. and Olsson, I. (1976). Granulopoiesis in chronic myeloid leukemia. II. Serial in vitro cloning of blood and bone marrow cells in agar culture. Blood 48, 351-360.
- Platt, K.L. and Oesch, F. (1977). An improved synthesis of trans-5,6-dihydroxy-1,3-cyclohexadiene (trans-1,2-dihydroxy-1,2-dihydrobenzene). Synthesis, 449-450.
- Rusch, G.M., Leong, B.K.J. and Laskin, S. (1977). Benzene metabolism. J. Tox. Env. Health suppl. 2, 23-36.
- Sammett, D., Lee, E.W., Kocsis, J.J. and Snyder, R. (1979). Partial hepatectomy reduces both metabolism and toxicity of benzene. J. Tox. Env. Health 5, 785-792.
- Sato, T., Fukuyama, T., Suzuki, T. and Yoshikawa, H. (1963). 1,2-Dihydro-1,2-dihydroxybenzene and several other substances in the metabolism of benzene. J. Biochem. 53, 23-27.
- Selling, L. (1916). Benzol as a leucotoxin. Studies on the degeneration of the blood and hematopoietic organs. Johns Hopkins Hosp. Rep. 17, 83-142.

Siou, M.G. and Conan, L. (1977). Activité mutagène du benzène et du benzo(a)pyrène: Mise en évidence par la technique des corps de Howell-Jolly (micronucleus test). Cah. Notes Doc. 69, 433-444.

Snyder, R., Uzuki, F., Gonasun, L., Bromfield, E. and Wells, A. (1967). The metabolism of benzene in vitro. Toxicol. Appl. Pharmacol. 11, 346-360.

Snyder, R. and Kocsis, J.J. (1975). Current concepts of chronic benzene toxicity. CRC Crit. Rev. Toxicol. 3, 265-288.

Steinberg, B. (1949). Bone marrow regeneration in experimental benzene intoxication. Blood 4, 550-556.

Tunek, A., Platt, K.L., Bentley, P. and Oesch, F. (1978). Microsomal metabolism of benzene to species irreversibly binding to microsomal protein and effects of modification of this metabolism. Mol. Pharmacol. 14, 920-929.

Tunek, A. and Oesch, F. (1979). Unique behaviour of benzene monooxygenase: activation by detergent and different properties of benzene- and phenobarbital-induced monooxygenase activities. Biochem. Pharmacol. 28, 3425-3429.

Tunek, A., Platt, K.L., Przybylski, M. and Oesch, F. (1980). Multi-step metabolic activation of benzene. Chem.-Biol. Interact., in press.

Uyeki, E.M., El Ash-kar, A., Shoeman, D.W. and Bisel, T.U. (1977). Acute toxicity of benzene inhalation to hemopoietic precursor cells. Toxicol. Appl. Pharmacol. 40, 49-57.

TABLE 1

Effects of phenol, hydroquinone and benzene dihydrodiol on mice CFU-C and marrow cellularity.

Experiment	CFU-C/tibia $\times 10^{-3}$	cells/tibia $\times 10^{-5}$	CFU-C/ 10^5 cells
1. control	6.6 ± 0.7	66.3 ± 7.4	100 ± 6
phenol	5.4 ± 0.3^a	64.8 ± 3.7^b	83 ± 5^a
2. control	5.6 ± 0.7	50.8 ± 6.2	111 ± 5
phenol	4.5 ± 0.3^a	56.0 ± 3.7^b	80 ± 4^c
hydroquinone	6.7 ± 0.6^b	53.3 ± 5.7^b	126 ± 14^b
dihydrodiol	5.5 ± 0.6^b	56.5 ± 5.8^b	98 ± 10^b
3. control	5.0 ± 0.6	64.3 ± 7.2	77 ± 8
hydroquinone	5.1 ± 0.7^b	56.9 ± 8.2^b	89 ± 10^a
dihydrodiol	3.9 ± 0.5^a	55.1 ± 7.5^a	70 ± 10^b

Phenol, hydroquinone and benzene dihydrodiol were given in 6 daily injections at a dose in each injection of 50 mg/kg body weight; all animals were given 100 μ l olive oil per injection. Experiments 1 and 2 contained 4 mice per group and experiment 3 had 8 mice per group. The values represent mean $\pm$ SD and were compared by Student's t-test.

^a $p < 0.05$

^b not significant

^c $p < 0.001$

TABLE 2

Effects of toluene and benzene on mice CFU-C and marrow cellularity.

Experiment	CFU-C/tibia $\times 10^{-3}$	cells/tibia $\times 10^{-5}$	CFU-C/ 10^5 cells
1. control	5.6 ± 0.7	50.8 ± 6.2	111 ± 5
toluene	3.6 ± 0.3 ^a	40.8 ± 2.9 ^b	89 ± 4 ^a
benzene	0.9 ± 0.1 ^c	15.0 ± 2.2 ^c	57 ± 4 ^c
tol. + benz.	3.5 ± 0.2 ^a	39.0 ± 2.5 ^b	90 ± 6 ^a
2. control	5.0 ± 0.6	64.3 ± 7.2	77 ± 8
toluene	5.1 ± 0.8 ^d	62.6 ± 9.5 ^d	81 ± 5 ^d
benzene	0.3 ± 0.1 ^c	6.8 ± 1.4 ^c	52 ± 7 ^a
tol. + benz.	1.9 ± 0.3 ^c	31.4 ± 4.9 ^c	61 ± 5 ^b

Toluene and benzene were given in 6 daily injections at doses in each injection of 867 and 440 mg/kg body weight, respectively; all animals were given 100 μ l olive oil per injection. Experiment 1 contained 4 animals per group and experiment 2 had 8 animals per group. The values represent mean $\pm$ SD and were compared by Student's t-test.

^a $p < 0.01$

^b $p < 0.05$

^c $p < 0.001$

^d not significant

TABLE 3

Effects of phenobarbital and benzene on mice CFU-C and marrow cellularity.

Experiment	CFU-C/tibia $\times 10^{-3}$	cells/tibia $\times 10^{-5}$	CFU-C/ 10^5 cells
1. control	5.6 ± 0.7	50.8 ± 6.2	111 ± 5
phenobarbital	4.0 ± 0.4 ^a	39.0 ± 3.7 ^b	103 ± 4 ^d
benzene	0.9 ± 0.1 ^c	15.0 ± 2.2 ^c	57 ± 4 ^c
phenob. + benz.	1.7 ± 0.3 ^c	15.3 ± 2.4 ^c	108 ± 3 ^d
2. control	11.6 ± 1.1	62.9 ± 5.8	185 ± 14
phenobarbital	12.3 ± 1.0 ^d	74.7 ± 6.2 ^a	165 ± 16 ^b
benzene	0.6 ± 0.3 ^c	3.9 ± 1.6 ^c	155 ± 20 ^a
phenob. + benz.	1.2 ± 0.3 ^c	8.6 ± 1.9 ^c	135 ± 14 ^c

Benzene was given in 6 daily injections at a dose in each injection of 440 mg/kg body weight and phenobarbital was administered in the drinking water at a concentration of 0.1%. All animals were given 100 μ l olive oil per injection. Experiment 1 contained 4 mice per group and experiment 2 had 8 mice per group. Values represent means $\pm$ SD and were compared by Student's t-test.

^a $p < 0.01$

^b $p < 0.05$

^c $p < 0.001$

^d not significant

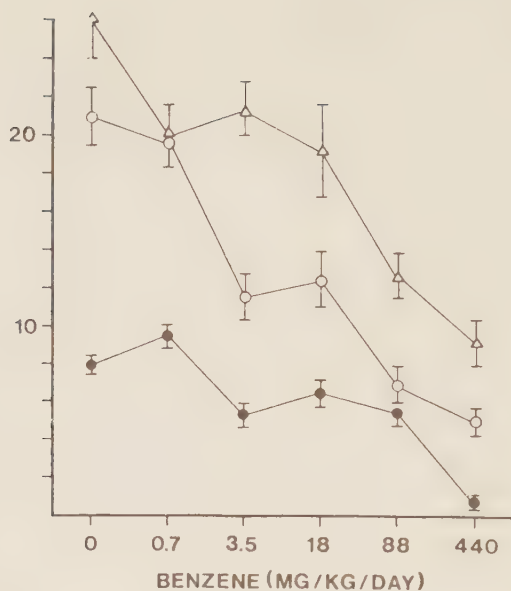


FIGURE 1

The effect of increasing doses of benzene on the concentration of CFU-C, cells/tibia and CFU-C/tibia of mouse bone marrow. The animals were given 6 daily injections and sacrificed the next day. Δ — Δ , CFU-C/10⁴ cells; $\bullet$ — $\bullet$, cells/tibia $\times 10^{-6}$; $\circ$ — $\circ$, CFU-C/tibia $\times 10^{-3}$. Values are mean $\pm$ SD (n = 4).

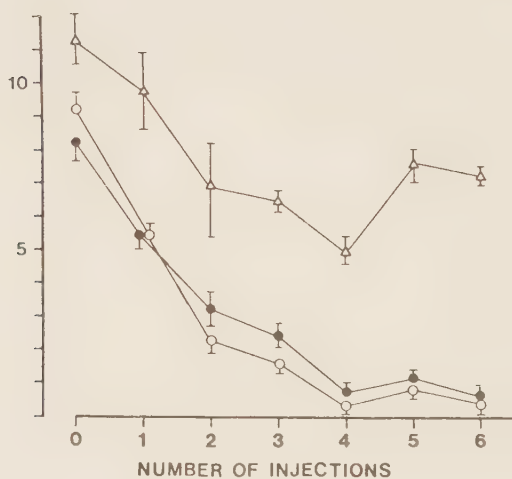


FIGURE 2

The effect of repeated daily injections of benzene (440 mg/kg/day) on the concentration of CFU-C, cells/tibia and CFU-C/tibia of mouse bone marrow. Δ — Δ , CFU-C/10⁴ cells; $\bullet$ — $\bullet$, cells/tibia $\times 10^{-6}$; $\circ$ — $\circ$, CFU-C/tibia $\times 10^{-3}$. Values are mean $\pm$ SD (n = 4).

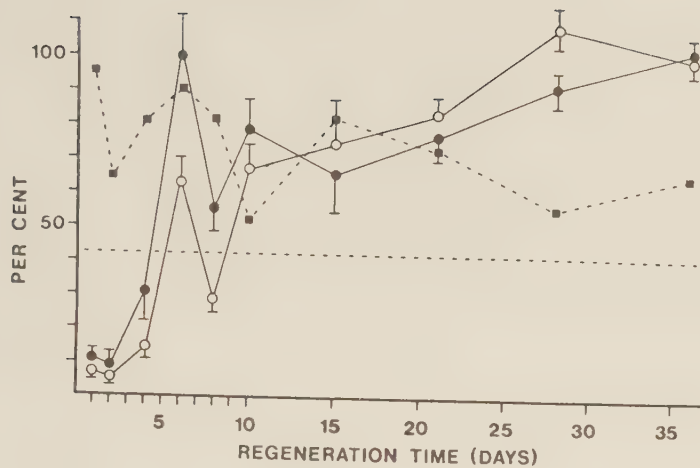


FIGURE 3

Regeneration of mouse bone marrow after 6 daily injections of 440 mg/kg benzene. ■---■, per cent of CFU-C in S-phase; ●—●, cells/tibia in per cent of controls; ○—○, CFU-C/tibia in per cent of controls. The dotted horizontal line indicates the normal value for CFU-C in S-phase ($42.2\% \pm 2.3$ SD). Values are mean $\pm$ SD ($n = 4$).

FOOTNOTES

1. This work was supported by the Swedish Cancer Society.
2. Abbreviations used: benzene dihydrodiol, 1,2-dihydro-1,2-dihydroxy benzene; CFU-C, colony forming units-culture (see Metcalf, 1973); SKF-525A, 2-diethylaminoethyl 2,2-diphenylpentanoate hydrochloride.

TOXIC EFFECTS OF BENZENE AND HYDROQUINONE ON BONE MARROW IN MICE.

A. Tunek, B. Högstedt and T. Olofsson.^a

Institute of Environmental Health, University of Lund, Sölvegatan 21,
223 62 Lund, Sweden and ^a Research Department C, E-blocket, University
Hospital, 221 85 Lund, Sweden.

Introduction.

Benzene is a hemotoxic agent, and much evidence indicates that it can induce leukemia in man (for reviews see references 1 and 2). The effects of benzene on the bone marrow were studied already in the beginning of this century, and it appears that virtually all cell lines in the marrow are affected by benzene exposure (1,3).

The mechanism for benzene toxicity has not been elucidated in detail. Most researchers agree, however, that benzene requires metabolic activation to exert its effects (2). Experiments with rat liver microsomes indicated that p-benzosemiquinone and/or p-benzoquinone, i.e. further metabolites of hydroquinone and phenol, were the main reactive metabolites of benzene (4,5). Several studies using cell cultures also indicated that dihydroxylated benzene metabolites, or further metabolites of these, were toxic (6,7,8). On the other hand, experiments where benzene or its metabolites have been administered to whole animals have in most cases revealed that the metabolites do not reproduce the severe hemotoxic effects of benzene (9,10,11).

Recently, we investigated the effects of benzene, phenol, benzene dihydrodiol and hydroquinone on the overall cellularity and on the amount of colony forming granulopoietic stem cells (CFU-C) in mouse bone marrow (11). Benzene gave strong toxic effects, but the metabolites, at the dose tested, gave only very mild toxicity. In the present study we have investigated the effects of the three benzene metabolites mentioned above over a wide dose range. In addition, we have studied the potential of benzene and these benzene metabolites to induce the formation of micronuclei in erythropoietic cells. Benzene has been shown by others to induce micronuclei (12,13,14), but no complete dose-response studies have been undertaken, and, to our knowledge, benzene metabolites have not been systematically tested in the micronucleus test. Furthermore, our intention was also to

compare the sequence of occurrence of different adverse effects following administration of benzene or its metabolites.

Materials and Methods.

The general outline of the experiments, injection of chemicals and culturing of bone marrow cells to determine the number of CFU-C/tibia were as detailed previously (11).

Bone marrow cells remaining after determination of cellularity and after starting cell cultures for determination of the number of CFU-C/tibia were prepared for the micronucleus test (15,16). The cells were pelleted by centrifugation at 1000 r.p.m. (MSE GF 6) for 10 minutes. Then they were suspended in a few drops of a mixture of equal parts of isotonic phosphate buffer, pH 7.0, and fetal calf serum. The cells were smeared on glasses, air dried, and then the smears were stained with May-Grunwald-Giemsa's stain. Only interphase cells were analyzed. The micronucleus in a polychromatic erythrocyte (PCE) was defined as a rounded, bluish or almost black, sharply outlined structure, about 1μ m across. For each group of mice 2000 PCE were examined.

The observers counting cells, colonies and micronuclei were not aware of which treatment the mice had received.

Results.

Effects of benzene.

The effects of various doses of benzene on cellularity and CFU-C/

tibia were recently investigated (11). In the present study the occurrence of micronuclei in PCE after identical benzene treatments has been evaluated. To facilitate comparison of the dose dependence of the development of the different adverse effects, data from (11) have been replotted in Fig. 1 and 2 together with new results obtained with the micronucleus test.

Benzene (each dose 440 mg/kg body weight) was injected subcutaneously at 1 - 6 daily doses, and the mice were sacrificed on the day after the last benzene injection. Fig. 1 shows that micronuclei were obtained already after a single injection, and that a maximum was reached after 3 injections. The reason why the number of micronuclei/2000 PCE was reduced after more than 3 doses was probably that the toxic effects were severe enough to cause cell death before the PCE-stage was reached during erythropoiesis. 4 - 6 benzene injections suppressed cellularity and number of CFU-C/tibia by up to 95 % (Fig. 1 and (11)).

In other experiments benzene doses ranging between 0.7-440 mg/day/kg body weight were given once a day for 6 consecutive days and the mice were sacrificed on day 7 (Fig. 2). Micronuclei were found only after the 2 highest doses (88 and 440 mg benzene/day/kg body weight). Cellularity and, particularly, CFU-C/tibia appeared to be considerably more sensitive to low doses of benzene, significant reductions being observed already at a dose of 3.5 mg benzene/day/kg body weight (Fig. 2 and (11)).

Effects of phenol and benzene dihydrodiol.

In the previous study we found no, or very mild, toxic effects of the two benzene metabolites phenol and benzene dihydrodiol on cellularity and CFU-C/tibia at 6 daily doses of 50 mg/kg body weight (11). In the present study we have tested phenol at 6 daily doses of up to 100 mg/kg body weight, and the dihydrodiol at 6 daily doses of up to 300 mg/kg for their effects on the 2 parameters mentioned,

and in the micronucleus test. In no case were any micronuclei induced, and the effects on cellularity and on CFU-C/tibia were none at all or very mild.

Effects of hydroquinone.

In the previous study (11) hydroquinone was tested for its effects on cellularity and CFU-C/tibia at 6 daily doses of 50 mg/kg body weight. No significant effects were achieved, although there was a tendency that the number of CFU-C/tibia was increased by the treatments. In the present study doses ranging from 20-100 mg hydroquinone/kg body weight, injected daily on 6 consecutive days, were tested. Hydroquinone did induce micronuclei in PCE at all doses above 6x20 mg/kg body weight (Fig.3). The frequency of micronuclei increased rather slowly up to a dose of about 6x50 mg/kg, then a sharp increase occurred and a maximum was obtained at 6x70-6x80 mg/kg. The cellularity was slightly elevated at low doses, was about the same as in untreated animals at 6x50 mg/kg, and was suppressed at higher doses (Fig. 3). The number of CFU-C/tibia followed a similar pattern as the cellularity. However, the increase at low and moderate doses was more pronounced (exception 6x20 mg/kg) and the suppression at high doses was less clear for CFU-C/tibia than for cellularity.

Discussion.

The data presented in this study clearly show that hydroquinone, as well as benzene, is a hemotoxic agent in mice. Rather high doses of hydroquinone were required to produce the effects, and the question arises if it is reasonable to draw the conclusion that formation of hydroquinone from benzene is an important factor for development of benzene toxicity. When measuring urinary metabolites, it is rare that more than 5% of a benzene dose is excreted as hydroquinone (1). Thus, a dose of 440 mg benzene/kg body weight

would result in excretion of about 20 mg hydroquinone/kg body weight. However, 6 injections of 20 mg hydroquinone/kg gave virtually no toxicity, while 6 doses of 440 mg benzene/kg made the marrow almost aplastic and induced high frequencies of micronuclei in PCE. There are, however, good reasons to believe that such a calculation is totally misleading. Firstly, evidence has been presented that metabolites of benzene accumulate in the bone marrow following exposure to benzene, and that these metabolites also are formed in the bone marrow (17,18). Secondly, since hydroquinone is a substrate for conjugation reactions, a large portion of a subcutaneously injected dose will probably be conjugated in the liver and in other organs and be excreted before reaching the bone marrow. Thus, we do not have enough information to compare doses of benzene in vivo with doses of hydroquinone in vivo. It may even be speculated that administration of benzene could be the most effective way to expose the bone marrow to hydroquinone and other benzene metabolites in vivo.

When comparing the effects of benzene and hydroquinone, some differences were observed. Benzene depressed the cellularity and, particularly, the CFU-C/tibia at doses considerably lower than those inducing micronuclei. Hydroquinone administration, on the other hand, resulted in formation of micronuclei at doses rather giving increased levels of cells and CFU-C/tibia. Furthermore, benzene, particularly at moderate doses, suppressed CFU-C/tibia more than the overall cellularity, while the opposite was true for hydroquinone at most doses. It is likely, therefore, that some other metabolite beside hydroquinone, or benzene itself, also possesses hemotoxic properties.

In summary, the data indicate that metabolic formation of hydroquinone from benzene is of importance for the development of the hemotoxic effects of benzene in mice in vivo, but other metabolites, or benzene itself, also probably contribute to the toxicity. These results indicate that the observations made in experiments with rat liver microsomes, showing p-benzosemiquinone and/or p-benzoquinone to be major reactive metabolites of benzene (4,5), may have

toxicological relevance.

Acknowledgement.

This research was supported by the Swedish Cancer Society and the Swedish Medical Research Council.

References.

1. Laskin, S. and Goldstein, B. D. J. Tox. Env. Health, suppl. 2, 1977.
2. Snyder, R. and Kocsis, J. J. CRC Crit. Rev. Toxicol., 3, 265, 1975.
3. Selling, L. Johns Hopkins Hosp. Rep., 17, 83, 1916.
4. Tunek, A., Platt, K. L., Bentley, P. and Oesch, F. Mol. Pharmacol., 14, 920, 1978.
5. Tunek, A., Platt, K. L., Przybylski, M. and Oesch, F. Chem.-Biol. Interact., in press.
6. Morimoto, K. and Wolff, S. Cancer Res., 40, 1189, 1980.
7. Diaz, M., Fijtman, N., Carricarte, V., Braier, L. and Diez, J. In Vitro, 15, 172 (abstract 23), 1979.

8. Harrison, K. and Randoll, F. W. Quart. J. exp. Physiol., 74, 141, 1948.
9. Mitchell, J. R. Fed. Proc., 30, abstract 2044, 1971.
10. Nomiyama, K. Ind. Health, 3, 53, 1965.
11. Tunek, A., Olofsson, T. and Berlin, M. submitted for publication.
12. Siou, M. G. and Conan, L. Cah. Notes Doc., 69, 433, 1977.
13. Lyon, J. P. Ph.D. Thesis, University of California, 1975.
14. Hite, M., Pecharo, M., Smith, I. and Thornton, S. Mutation Res., 77, 149, 1980.
15. Schmid, W. Mutation Res., 31, 9, 1975.
16. Jenssen, D. and Ramel, C. Mutation Res., 41, 311, 1976.
17. Rickert, D. E., Baker, T. S., Bus, J. S., Barrow, C. S. and Irons, R. D. Toxicol. Appl. Pharmacol., 49, 417, 1979.
18. Andrews, L. S., Lee, E. W., Witmer, C. M., Kocsis, J. J. and Snyder, R. Biochem. Pharmacol., 26, 293, 1978.

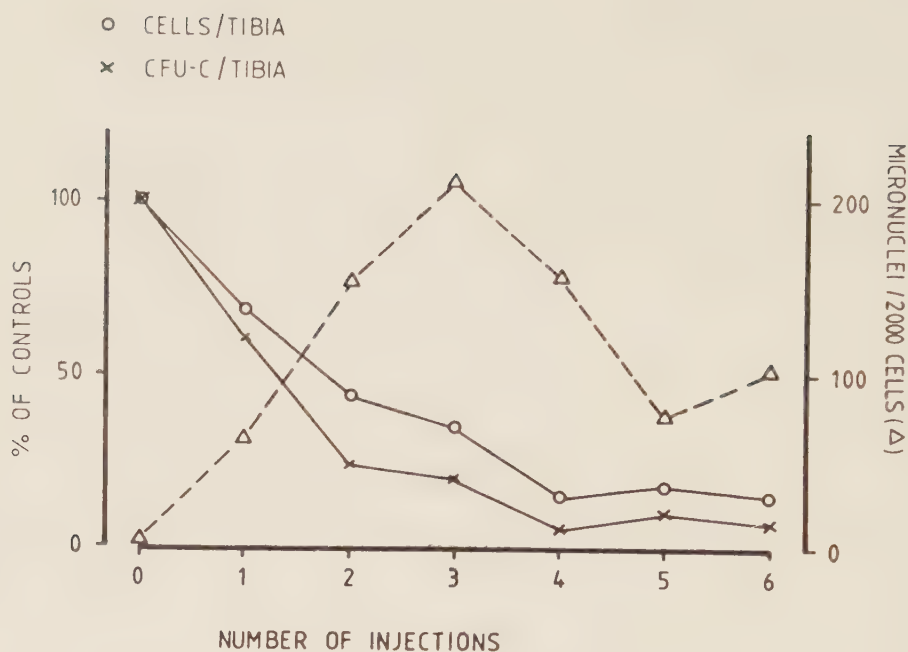


Fig. 1. The effect of repeated injections of benzene (440 mg/kg body weight) on cellularity (○, the value observed in animals not receiving benzene (8.1×10^6 cells/tibia) defined as 100 %), CFU-C/tibia (×, the value observed in animals not receiving benzene (9.1×10^3 CFU-C/tibia) defined as 100 %), and micronuclei/2000 PCE (Δ).

The animals were sacrificed on the day after the last benzene injection. The benzene was diluted in 100 μ l olive oil, and all animals received the same amount of oil injected ($6 \times 100 \mu$ l). The values given for cellularity and CFU-C/tibia are means ($n=4$), and the standard deviations were less than 20 %.

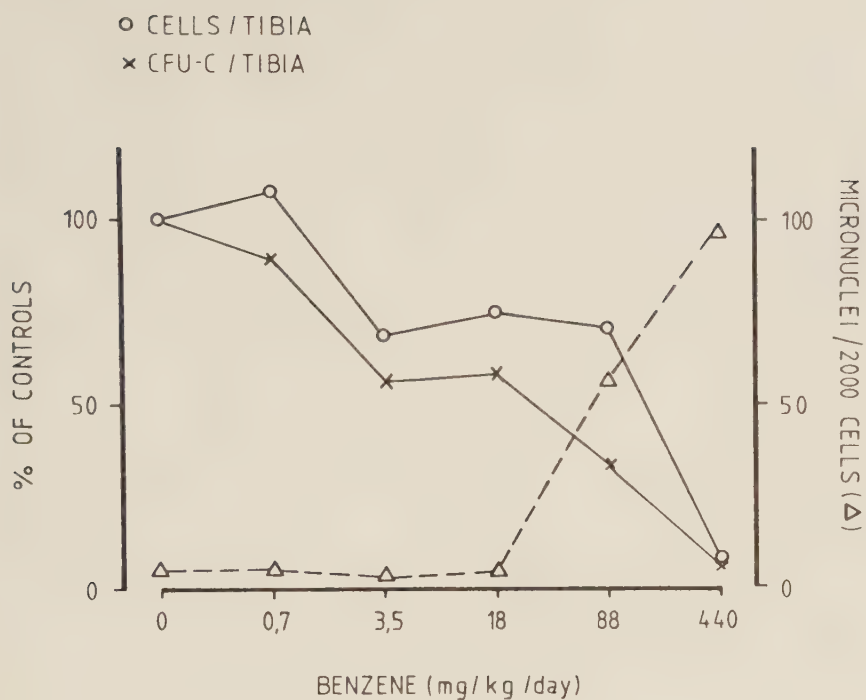


Fig. 2. The effect of increasing doses of benzene on cellularity (○ , the value observed in animals not receiving benzene (8.0×10^6 cells/tibia) defined as 100 %), CFU-C/tibia (× , the value observed in animals not receiving benzene (21×10^3 CFU-C/tibia) defined as 100 %), and micronuclei/2000 PCE (Δ).

The animals were injected with the doses indicated on 6 consecutive days and sacrificed on day 7. Each animals received identical amounts of olive oil ($6 \times 100 \mu\text{l}$). The values given for cellularity and CFU-C/tibia are means ($n=4$), and the standard deviations were less than 15 %.

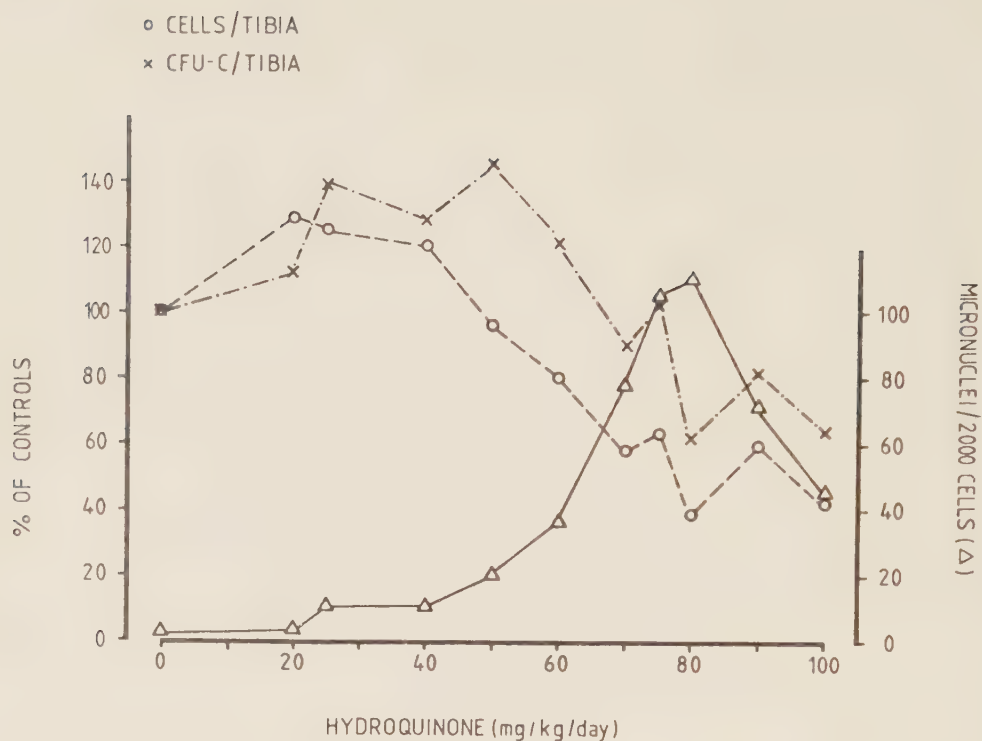


Fig. 3. The effect of increasing doses of hydroquinone on cellularity (○, the value observed in animals not receiving hydroquinone (7.1×10^6 cells/tibia) defined as 100 %), CFU-C/tibia (×, the value observed in animals not receiving hydroquinone (15.4×10^3 CFU-C/tibia) defined as 100 %), and micronuclei/2000 PCE (Δ).

The animals were injected with the doses indicated on 6 consecutive days and sacrificed on day 7. The hydroquinone was dissolved in physiological saline, and each animal received $6 \times 100 \mu\text{l}$ saline. The values given for cellularity and CFU-C/tibia are means ($n=4$), and the standard deviations were less than 19 %.

